UNIVERSITY OF LIVERPOOL

MODULATION OF DENDRITIC CELL SIGNALLING AND FUNCTION BY REDOX REGULATORS

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

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DECLARATION

This thesis is the result of my own work. The material contained within this thesis has not been presented, nor is currently being presented, either wholly or in part for any degree or other qualification.

Laith Mohammed Abbas Al-Huseini

This research was carried out in the MRC Centre for Drug Safety Science,
Department of Pharmacology and Therapeutics,
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For My Family

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ABSTRACT

Dendritic cells (DCs) are antigen-presenting cells crucial for the initiation and coordination of primary adaptive immune responses. Immature DCs (iDCs) express low levels of MHC class II and co-stimulatory molecules such as CD80, CD86, and CD40, with high phagocytic capacity and limited ability to induce antigen-specific T cell activation. DC maturation is associated with up-regulation of co-stimulatory molecules and cytokine production, rendering the DCs competent in T cell activation and the elicitation of an immune response. DC function and co-stimulatory receptor gene expression are known to be regulated by intracellular redox status, NF-κB and MAPKs signalling pathways.

Intracellular reactive oxygen species (ROS) levels influence DC maturation and function. The transcription factor, Nrf2, is essential for maintaining intracellular redox homeostasis. In response to oxidative stress, Nrf2 induces the transcription of a set of cytoprotective and antioxidant genes, including heme oxygenase-1 (HO-1), that are required for detoxification of xenobiotics and their reactive metabolites and the nullification of oxidative insult.

It is now emerging that Nrf2, and its gene product, HO-1, play pivotal roles in regulation of immune responses. However, the key signalling mechanisms involved in Nrf2 and HO-1-mediated altered DC function has not been fully elucidated and requires further investigation. In addition, the role of ROS in the absence of Nrf2 or HO-1 activity, in DC activation and function has not been investigated.

Using immature bone marrow-derived DCs (iDCs) from $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice, we demonstrate in the first part of the work presented in this thesis, that Nrf2 deficiency in iDCs resulted in increased ROS levels, enhanced iDCs co-stimulatory receptor expression, and increased iDC-mediated antigen-specific CD8 T cell stimulatory capacity in response to an antigenic peptide.

Using antioxidant vitamins to reset ROS levels in $Nrf2^{-/-}$ iDCs, we show that elevated ROS was not responsible for the altered phenotype and function of these DCs. Additionally, using appropriate pharmacological inhibitors, we demonstrate that the altered $Nrf2^{-/-}$ iDC

phenotype and function did not require NF-κB, ERK or JNK activity but was dependent on p38MAPK-CREB/ATF1 activity.

Based on these experimental results, we conclude that Nrf2 regulates DC maturation and function by modulating intracellular signalling pathways independent of intracellular ROS levels.

In the second part of the study, we demonstrate that inhibition of HO-1 activity in iDCs resulted in DCs with raised intracellular ROS levels, a mature phenotype, impaired phagocytic and endocytic function, and increased capacity to stimulate antigen-specific CD8 T cells. Interestingly, our results reveal that the increased ROS levels following HO-1 inhibition did not underlie the changes in phenotype and functions observed in these iDCs. Importantly, we show that the p38MAPK-CREB/ATF1 pathway was involved in the mediation of the phenotypic and functional changes arising from HO-1 inhibition. Furthermore, up-regulation of HO-1 activity rendered iDCs refractory to lipopolysaccharide-induced activation of p38MAPK-CREB/ATF1 pathway and DC maturation. Finally, we demonstrate that treatment of iDC with the HO-1 substrate, heme, recapitulated the effects that result from HO-1 inhibition. Based on these experimental results, we conclude that HO-1 regulates DC maturation and function by modulating the p38MAPK-CREB/ATF1 signalling axis.

Collectively, the work described in this thesis highlights the importance of the redox regulators, Nrf2 and HO-1, in controlling DC immune functions. This work supports the basis for utilisation of Nrf2 and HO-1 as potential molecular targets for pharmacological intervention in disease states that arise from dysregulated redox function and the design of new pharmacological strategies aimed at modulating DC function in the therapy of immune diseases.

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PUBLICATIONS

Papers

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Laith M A Al-Huseini, Han Xian Aw Yeang, Junnat Hamdam, Christopher Goldring, Jean G Sathish. Cellular redox affects dendritic cells maturation and phagocytic function. 6th European Network of Immunology Institutes Summer School in Advanced Immunology, Sardinia, Italy, May, 2011. Poster presentation

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Laith M A Al-Huseini, Swaminathan Sethu, Junnat Hamdam, Naif Alhumeed, Han Xian Aw Yeang, Christopher Goldring, Kevin Park, Jean G Sathish. The Redox-responsive transcription factor Nrf2 is required for optimal p38MAPK signalling in dendritic Cells. British Pharmacology Society Winter meeting, London, UK, December, 2012. Poster presentation

Laith M A Al-Huseini, Swaminathan Sethu, Han Xian Aw Yeang, Junnat Hamdam, Naif Alhumeed, Thaventhiran Thilipan, Christopher Goldring, Kevin Park, Jean G Sathish. Elevated intracellular ROS is not responsible for altered phenotype and function of Nrf2 deficient dendritic cells. WORLD IMMUNE REGULATION MEETING – VII, Davos, Switzerland, March, 2013. Poster presentation

Abbreviations

2-ME	2-Mercaptoethanol
ANOVA	analysis of variance
AP-1	activator protein 1
APC	Antigen presenting cell
APL	altered peptide ligand
APS	ammonium persulphate
ARE	antioxidant response element
ASK1	apoptosis signal-regulating kinase 1
ATP	adenosine triphosphate
ATF1	activating transcription factor 1
BMDC	bone marrow– derived dendritic cell
bp	base pair
BSA	bovine serum albumen
bZip	basic leucine zipper
C	Celsius
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation (cell surface molecules expressed on various cell types)
cDNA	complementary deoxyribonucleic acid
CFSE	carboxy fluorescein succinimidyl ester
c/EBPβ	CCAAT- enhancer binding proteins
CNC	cap 'n' collar
CIITA	class II major histocompatibility complex transactivator
CO_2	carbon dioxide
ConA	Concanavalin A
COOH	carboxyl
CoPPIX	cobalt protoporphyrin IX
cpm	counts per minute
CRE	Cre recombinase
CREB	Cyclic-AMP response element binding protein
CSA	Cyclosporin A
Csk	Carboxy terminal Src Kinase
cSMAC	central supramolecular activation cluster
CTL	cytotoxic T lymphocyte
Da	Dalton
DAG	Diacyl Glycerol
DC	dendritic cell
DMSO	dimethyl sulphoxide
DN	double negative ($CD4^-CD8^-$)
DNA	deoxyribonucleic acid
DP	double positive ($CD4^+CD8^+$)
DPP3	dipeptidyl-peptidase 3
dsRNA	double stranded RNA
Dusp	Dual specificity phosphatase

ECH	Embedded contact homology
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	extracellular signal regulated protein kinase
EtOH	Ethanol
Fab	Ig-variable region
FACS	fluorescence activated cell sorting
FasL	Fas ligand
FBS	fetal bovine serum
Fc	Ig-constant region
FCS	fetal calf serum
FITC	fluorescein isothiocyante
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCL	γ-glutamylcysteine ligase
GDP	guanosine diphosphate
GM-CSF	granulocyte-macrophage colony stimulating factor
GPX	GSH peroxidase
GSH	Reduced glutathione
GSR	GSH reductase
H_2O	water
H_2O_2	hydrogen peroxide
HBSS	Hanks balanced salt solution
HCl	Hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
HLA	Human leukocyte antigen
HO-1	heme oxygenase 1
HRP	horseradish peroxidase
HSF	heat shock factor
HSP	heat shock protein
IFN	Interferon
Ig	immunoglobulin
IκB	inhibitor of NF-κB
IKK	IκB kinase
IL	interleukin
IL-2	interleukin-2
IL-10	interleukin-10
iNOS	inducible nitric oxide synthase
IP	immunoprecipitation
IS	immunological synapse
ITAM	immunoreceptor tyrosine-based activation motif
IU	international units
IκB	inhibitor of κB
JNK	c-Jun amino-terminal kinase
kDa	Kilo Dalton
KO	Knock out
kV	Kilo volts

L	liter
LPS	lipopolysaccharide
µ	micro
ml	millilitre
M	molar
mA	milliamps
mAb	monoclonal antibody
MAPK	mitogen activated protein kinase
MAPKAPK	MAPK-activated protein kinase
$MgSO_4$	Magnesium sulfate
MHC	major histocompatibility complex
MHz	Mega Hertz
MFI	mean fluorescence intensity
min	minutes
MKPs	MAPK phosphatases
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger RNA
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
NADPH	nicotinamide adenine dinucleotide phosphate
Neh	Nrf2-ECH homology
NES	nuclear export signal
NF-AT	nuclear factor of activated T cells
NF-E2	nuclear factor erythroid 2
NFκB	Nuclear Factor Kappa Beta (nuclear factor kappa-light-chain-enhancer of activated B cells)
NH_4	Ammonium
NK	natural killer
NP-40	nonidet P-40
Nrf2	Nuclear Factor-Erythroid 2 (NF-E2) p45-related Factor-2
OD	Optical density
OD570nm	optical density at 570 nm
OH	hydroxyl
PBMC	Peripheral blood mononuclear cell
PBS	phosphate buffered saline
PD-1	programmed death-1
PE	phycoerythrin
PHA	Phytohemagglutinin
pI	isoelectric point
PI	propidium iodide
pKa	acid dissociation constant
PKA	Protein kinase A
PKC	protein kinase C
PLCγ	phospholipase C gamma 1
PMA	phorbol 12-myristate-13-acetate
PRX	Peroxiredoxin

PS	Phosphatidylserine
PTB	phosphotyrosine binding
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
RCF (g)	Relative Centrifugal Force
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
rpm	revolutions per minute
SD	standard deviation of the mean
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-Polyacrylamide gel electrophesis
SH	Sulphydryl
siRNA	short interfering RNA
STAT1	signal transducers and activators of transcription 1
Std	Standard
TAP	transporter associated with antigen processing
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline-Tween
TCR	T cell receptor
TH	T-helper
TLR4	Toll-like receptor-4
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumor necrosis factor
TNF-R	TNF receptor
TNF-α	tumour necrosis factor α
TRAF6	TNF receptor associated factor 6
TRX	Thioredoxin
TRX-R	TRX reductase
Tyr	Tyrosine
U	Unit
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
V	Volts
v/v	volume/volume
w/v	weight/volume
WT	Wild Type
γ	Gamma
ZnPPIX	zinc protoporphyrin IX

CHAPTER ONE

INTRODUCTION

1.1 Immunity

Immunity is broadly defined as the organism's ability to protect itself from invading pathogens. Immunity is mediated by the coordinated response of a large variety of immune cells and soluble mediators. Checks and balances also exist within the immune system that operate to prevent an attack against the body's own constituents, a phenomenon termed tolerance. Immunity can be generally categorized into innate and adaptive (specific or acquired) immunity.

Innate immunity represents the initial rapid defensive response against encountered microbes. This was previously believed to be a non-specific immune response, characterised by the engulfment and digestion of microorganisms and foreign substances via myeloid phagocytic cells. However, recent advances in immunology showed that innate immunity has considerable specificity in discriminating between pathogens versus host components and that it can be a prerequisite for the triggering of adaptive immunity. Innate immunity is present from birth and includes physical barriers (e.g. intact skin and mucous membranes), chemical barriers (e.g. gastric acid, digestive enzymes and bacteriostatic fatty acids of the skin), phagocytic cells, antigen presenting cells and the complement system (Beutler, 2004; Kimbrell and Beutler, 2001).

The fundamental functional distinction between innate and adaptive immune cells is the acquisition of memory by adaptive immune cells (but not by innate cells) which is specific to a single antigen or to a group of closely related antigens (Lo et al., 1999). Adaptive immunity is characterised by specificity and memory with slow onset in comparison to the rapid innate arm and is mediated by T and B lymphocytes.

1.2 Innate immune cells

Innate immune response is mostly dependent upon cells of myeloid origin that can engulf and destroy pathogens. While these cells have independent capabilities to deal with such infectious challenges, they have also evolved to be more effective in conjunction with cells and proteins of the adaptive immune system. For example, lymphoid cells of the adaptive immune system produce antibodies that opsonise bacteria which can then be destroyed by innate immune cells (Beutler, 2004).

These myeloid cells include mononuclear and polymorphonuclear phagocytes. The mononuclear phagocytes are the macrophages and monocytes and under inflammatory conditions monocytes can also mature into a subtype of dendritic cells which are discussed in section 1.3. The mononuclear phagocytes are competent at presenting antigens to T cells of the adaptive immune system. Macrophages are widely distributed throughout the body mostly in the lining parenchyma of major organs (e.g. heart, brain, lung, and liver) where they come in contact with invasive pathogens. They are morphologically diverse with specialised tissue-resident cell types. Dendritic cells follow similar distribution and function and also exhibit a variety of morphologies (e.g. the Langerhans cell of the skin; the plasmacytoid dendritic cell of the spleen). Although they exist as a minority population among the mononuclear phagocytes, DCs are capable of initiating adaptive immune responses to most pathogens by presenting a wide range of antigens to $CD4^+$ and $CD8^+$ T cells (Banchereau and Steinman, 1998).

The polymorphonuclear phagocytes include neutrophils, basophils, and eosinophils. They are of importance in the control of infection as they have a specialised ability to destroy microbes by a broad array of effector mechanisms including the production of pro-inflammatory mediators (Herant et al., 2003; Legrand et al., 2008; Wedemeyer et al., 2000).

The innate immune system also contains cells that are of a lymphoid origin called natural killer cells (NK cells) that are morphologically similar to lymphocytes. Natural killer cells target and lyse abnormal/viral-infected cells which have low/absent expression of MHC class I cell surface molecules with no prior specific sensitization of the host (unlike T cells). They play important roles in anti-tumour and anti-viral immunity (Ljunggren and Karre, 1990).

Dendritic cells are one of the crucial components of the immune system due to their essential role in the induction and control of T cell mediated immunity, as well as in the modulation of responses elicited by B cells and NK cells.

1.3 Dendritic cells (DCs)

Dendritic cells are professional antigen processing cells (APCs) with characteristic abilities in the acquisition, processing, and presentation of antigens to naive T cells in order to produce an adaptive immune response; therefore, they represent critical mediators of both immunity and tolerance (Banchereau and Steinman, 1998). They possess long membrane extensions that resemble dendrites of nerve cells (hence the name), which allow them to intimately interact with antigens and other cells. Dendritic cells play an important role in linking the innate arm of the immune system to the adaptive immune system. They do this by their exclusive ability to induce the differentiation of naïve T cells into effector or memory cells each with its unique function and cytokine profile (Banchereau et al., 2000). The importance of DCs is not only focused on their ability to induce competent immune responses against harmful pathogen-derived antigens (e.g. peptides derived from virus and bacteria), but also to avoid autoimmunity or provide tolerance to self-antigens (Guermonprez et al., 2002).

1.3.1 Origin and development of DCs

Dendritic cells develop from myeloid or lymphoid precursors (Ardavin et al., 2001). Dendritic cells were considered previously to be solely of myeloid origin; however, increasing evidences have shown that DCs can also be generated from lymphoid progenitors (Karsunky et al., 2003). Differentiation and development of haematopoietic stem cells (HSCs), during the process of haematopoiesis, gives rise to common myeloid precursors (CMPs) and common lymphoid precursors (CLPs) as shown in **Figure 1.1**.

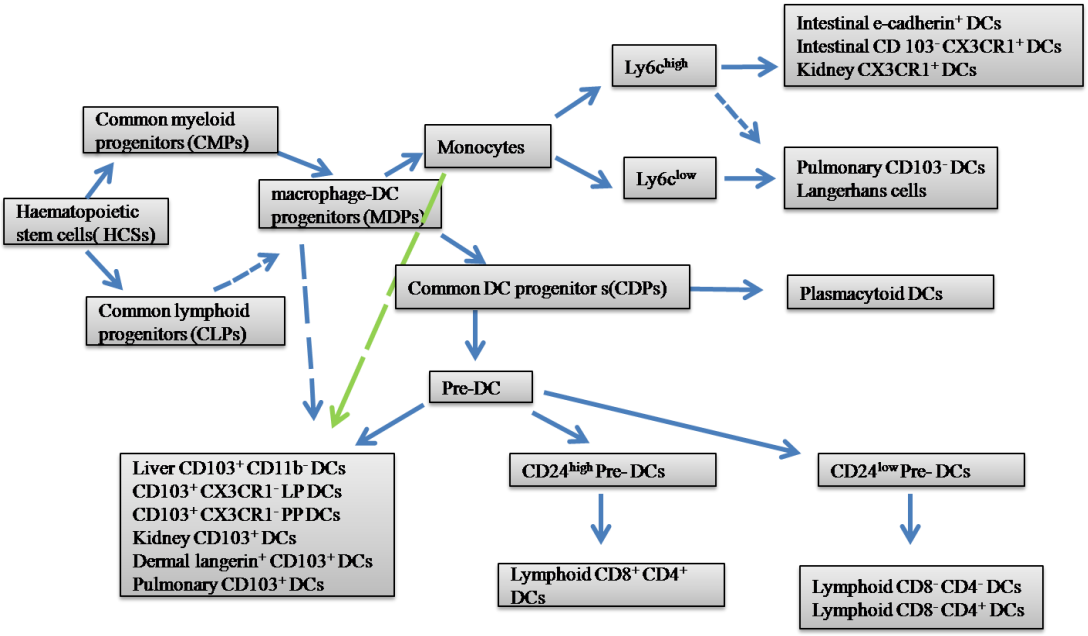


Figure 1.1. Dendritic cells (DCs) differentiation from pluripotent haematopoietic stem cells (HSCs). In the bone marrow, the HSCs differentiate into common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs). CMPs, within bone marrow, can be further differentiated into monocytes and pre-DCs. Monocytes and pre-DCs subsequently leave the bone marrow and enter the blood stream and migrate to lymphoid organs and peripheral tissues, where they differentiate into lymphoid DCs and tissue-resident DCs. In addition to CMPs, CLPs also have the potential to give rise to DCs, but their contribution is not well understood. Adapted from Kushwah and Hu 2011.

Macrophages, monocytes, granulocytes, megakaryocytes and erythrocytes originate from CMP, whilst NK cells, T cells and B cells originate from CLP (Akashi et al., 2000; Kondo et al., 1997). CMPs give rise to macrophage and dendritic cell precursors (MDPs) and these further differentiate into common DC-restricted progenitors (CDPs) (Cravens and Lipsky, 2002). Additional differentiation of CDPs yields either plasmacytoid DC (pDC) or pre-DC, which then migrate to various lymphoid tissues in order to differentiate into myeloid DC subsets. The differentiation into these subsets is programmed to be specific to particular tissue environments (Ardavin, 2003). Selective expression of cell surface proteins (markers) allows us to distinguish the multitude of DC subsets. For example, human pDCs express different cell markers such as CD123, BDCA-2, and BDCA-4, but do not express CD11c, while in mice pDCs are positive for CD11c, B220, PDCA-1 and Siglec-H (Kvale et al., 2006).

In order to study the function of DCs, many protocols have been developed to generate DCs *in vitro* from both human and murine cell sources. *In vitro* generation of DCs requires the culturing of progenitor cells present in the bone marrow and peripheral blood in the presence of cytokines like GM-CSF, IL-4, Flt3L, TNF-α and TGF-β (Lutz et al., 1999; Merad and Manz, 2009). For example, mouse bone marrow-derived DCs can be obtained *in vitro* by culturing bone marrow precursors with GM-CSF for 7-12 days with frequent feedings at days 3, 6, 8 and 10. The resultant DC has a phenotype of $CD11c^{+}$, $CD8^{-}$, MHC class II^{low} and $CD86^{low}$ (Lutz et al., 1999). Mouse DCs can also be isolated from spleen, a procedure which involves tissue digestion resulting in a DC population expressing MHC class II^{high} and $CD8^{+}$ (Vremec et al., 1992). Human blood monocytes can be differentiated *in vitro* into cells expressing an identical phenotype and functional features of blood DCs under the effect of a triple combination of IL- 4, IFN-γ and GM-CSF (Xu et al., 1995) or from human cord blood $CD34^{+}$ hematopoietic progenitors cultured with GM-CSF and TNF-α (Caux et al., 1996).

DCs possess a significant functional flexibility for either inducing immunity or tolerance. The induction of tolerance versus immunity is dependent on distinct DC development and activation states (Lutz and Schuler, 2002). Such induction is predicated on the two-signal model of T cell stimulation, in which a productive T cell immune response requires specific recognition of major histocompatibility complex (MHC)/peptide complexes by the T cell receptor (TCR) (signal 1) along with signalling through co-stimulatory molecules (signal 2). Cytokines produced by activated DCs help to shape the resultant T cell immune response (signal 3). There is cumulative evidence to indicate that immature myeloid and plasmacytoid DCs (see section 1.3.2.1) induce T cell tolerance (Jonuleit et al., 2001; Mahnke et al., 2002), whereas mature DC types (see section 1.3.2.2) have been shown to induce immunity (Cella et al., 1997b; Cerundolo et al., 2004).

1.3.2 Immature versus mature DCs

Based on their phenotypic and functional state (depending on their shape, antigen acquisition capacity, cell surface markers expression and antigen specific T cell activation; see **Figure 1.2**), DCs exist in two distinct forms: immature DCs (iDCs) and mature DCs (mDCs) (Banchereau et al., 2000).

1.3.2.1 Immature DCs

Immature DCs reside in the body's peripheral tissues (that are in contact with the external environment) such as the skin, the lining of nose, lungs, and the gastrointestinal tract (Banchereau et al., 2000). They detect pathogens, tissue damage and inflammation, and serve as sentinels and information carriers. Immature DCs exhibit a highly organized cytoskeleton, focal adhesion plaques, and slow motility with specialisation in capturing and processing antigens. These cells can efficiently capture pathogens, apoptotic cells, and antigens from the

environment through phagocytosis, macropinocytosis, or endocytosis. Immature DCs express low levels of MHC class II and co-stimulatory molecules (CD40, CD80, and CD86) on their surface (as shown in **Figure 1.2**), hence they are considered to be unable to present antigens efficiently to T cells and initiate an effective immune response (Banchereau et al., 2000). In steady-state conditions, DCs maintain themselves in the peripheral tissue in an immature state and they express low amounts of MHC and co-stimulatory molecules. Immature DCs that migrate to lymphoid tissues and present self-antigens to T cells induce T cell anergy instead of T cell activation which forms a basis for tolerance (Hawiger et al., 2001).

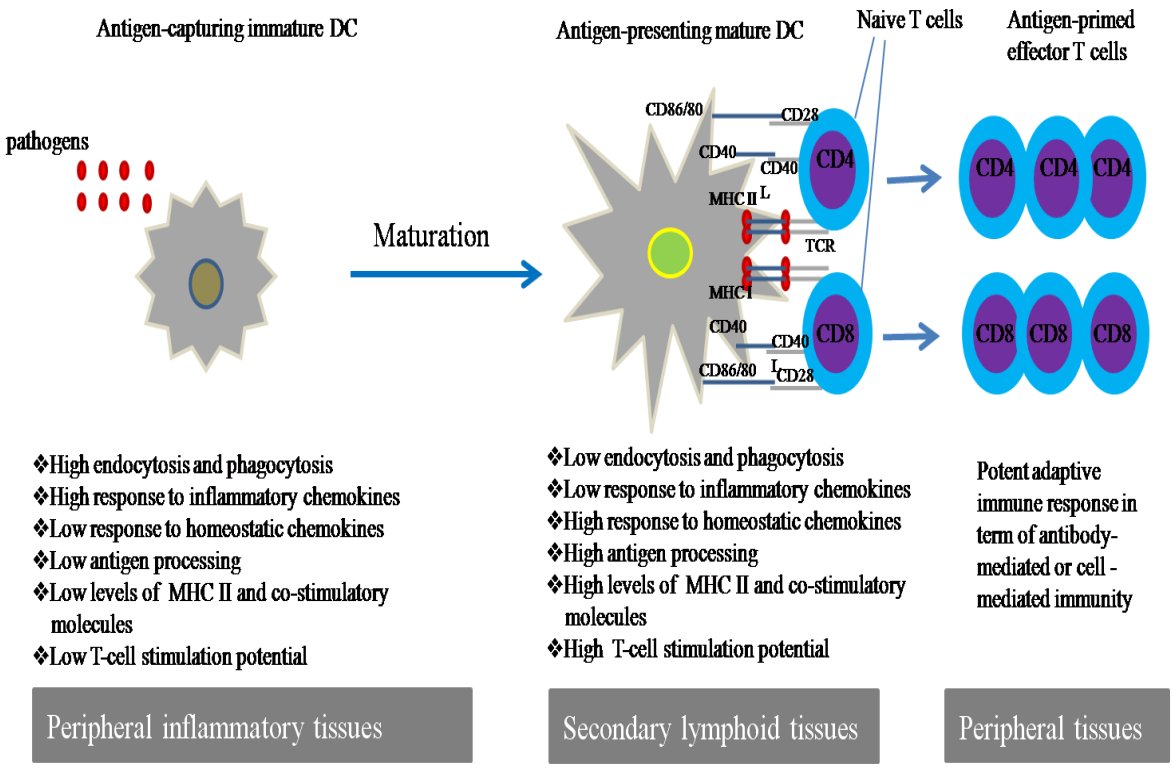


Figure 1.2. Dendritic cells (DCs) have central role in the immune system by generating antigen-specific T cells from naïve T cells. DCs sense and take up antigens via various PRRs in peripheral tissues, process them intracellularly into proteolytic peptides, and load these peptides onto major histocompatibility complex (MHC) class I and II molecules and become mature DCs. Subsequently, mature DCs migrate to secondary lymphoid organs and become competent to present antigens to T lymphocytes to generate effector T cells. Adopted from Sato 2007.

1.3.2.2 Mature DCs

Maturation of DCs is a process initiated by the engagement of their surface receptors such as Toll-like receptors, Fc receptors, cytokine receptors or CD40 with their respective ligands (Ardavin, 2003). Mature DCs highly express MHC class II and co-stimulatory molecules (CD80, CD86, and CD40) on their surface and this is associated with high antigen presentation and T cell activating ability (Banchereau and Steinman, 1998). Dendritic cell maturation is also accompanied by morphological changes including rearrangement of cytoskeleton proteins, loss of adhesive structures and possession of high cellular motility. This is accompanied with down-regulation of chemokine receptors that bind inflammatory chemokines and an up-regulation of the chemokine receptor 7 (CCR7) that binds to the chemokines expressed on T cells in lymphoid tissue (Hawiger et al., 2001; Winzler et al., 1997). The mature DCs migrate from the peripheral tissues through the afferent lymphatics towards the T cell zones of draining lymphoid organs where they have the ability to activate antigen-specific cytotoxic or helper T cells and induce their differentiation (Cella et al., 1997b). DC maturation is accompanied by secretion of a wide array of cytokines which have a significant influence on the elicited immune response. The secreted cytokine profile depends on the antigens or microbe product originally encountered by the DC. The cytokine milieu is critically involved in selectively directing T cell responses, known collectively as T cell polarisation. These instructive cytokines are produced in the early phases of infection and are required for the generation of appropriate immune responses to bacteria, virus, fungi or parasite infections (Langenkamp et al., 2000).

1.4 Antigen acquisition by DCs

Immature DCs are very efficient at capturing extracellular antigens and molecules through several mechanisms. This include: receptor-mediated endocytosis via C-type lectin receptors (mannose receptor and DEC-205) (Figdor et al., 2002; Jiang et al., 1995; Sallusto et al., 1995a), macropinocytosis (uptake of large vesicles of 0.5-3 μm) mediated by membrane ruffling driven by the actin cytoskeleton or macropinocytosis by uptake of small vesicles via clathrin-coated pits (Sallusto et al., 1995a), phagocytosis of bacteria, apoptotic and necrotic cell fragments and particulate matter via Fcγ receptor types I (CD64) and II (CD32) and scavenger receptor (CD36) (Albert et al., 1998; Fanger et al., 1996; Greenberg et al., 2006; Rescigno et al., 1999). Endocytosis is measured experimentally through DC uptake of fluorescently labelled particles such as dextran beads (Sallusto et al., 1995a). Phagocytosis of foreign antigens is enabled through engagement of DC receptors such as Fcγ, scavenger receptors, pathogen-specific receptors (e.g. Toll-like receptors), lectins or complement receptors. This is accompanied by actin-dependent internalization processes and rearrangement of the plasma membrane resulting in the formation the phagosome (Jutras and Desjardins, 2005; Niedergang and Chavrier, 2004). The remodelling of the cytoskeleton involves key players that include small Rho GTPases such as Rac, Cdc42 and RhoA (Hall, 1998a). The rate of DC phagocytosis can also be influenced by receptor systems such as TLR or CCR7 engagement both of which enhance phagocytosis (Doyle et al., 2004; Kikuchi et al., 2005; West et al., 2004b). While phagocytosis of pathogens by DCs delivers antigens to T cells, phagocytosis of apoptotic cells by iDCs suppress immune responses and induce self-tolerance by preventing the leaking of inflammatory intracellular components as well as through presentation of self-peptides to T cells (Banchereau et al., 2000).

1.5 Dendritic cell antigen presentation – role of MHC Class I and II

Dendritic cells (DCs) initiate immune responses through activation of naive T lymphocytes. This process requires DCs to take up antigens and cleave them into peptides, which are loaded onto highly polymorphic MHC molecules and presented to antigen-specific T lymphocytes. On mDCs, antigenic peptides bound to MHC class II initiates immunogenic responses by $CD4^+$ T lymphocytes (Banchereau and Steinman, 1998), while antigenic peptides bound to MHC class I enable mDCs to activate $CD8^+$ T lymphocytes and generate effector cytotoxic T lymphocytes (CTLs) that possess anti-viral and anti-tumour activity (Chai et al., 1999; Kemball et al., 2006).

1.5.1 MHC class I

MHC class I molecules are expressed on the surface of virtually all nucleated cells including DCs where they serve as the target antigens for $CD8^+$ T cells. Their main structure consists of a membrane-integrated glycoprotein (α chain), a small soluble protein, β2 microglobulin (β2m) with a binding groove that accommodates a short peptide usually of 8–10 amino acids (antigenic or self peptides) (Pamer and Cresswell, 1998). Endogenous or captured antigens are degraded by the ubiquitin-proteasome pathway, to generate peptides with appropriate length (8- to 10-amino acids) for the transporters associated with antigen processing (TAP) that matches distinct peptide preferences of different MHC class I molecules (Rock and Goldberg, 1999). This process is followed by the loading of peptides onto newly synthesized MHC class I molecules within the endoplasmic reticulum. DCs can load antigenic peptides on their MHC class I molecules through both an endogenous (generated in the cytosol by degradation of cytosolic proteins) and an exogenous pathway (through cross-presentation) and present them on the cell surface to $CD8^+$ cytotoxic T cells (Rock and Goldberg, 1999).

1.5.2 MHC class II

MHC class II proteins are polymorphic cell surface proteins which present foreign antigens to the T cell receptor of T helper cells. They are heterodimers of two polypeptide chains (α and β peptide chains) both of which span the membrane. These peptide chains are synthesized in the endoplasmic reticulum (ER), where they associate with the invariant chain (Ii) which protects the peptide-binding groove of the MHC class II heterodimer from being prematurely filled with self-proteins. MHC class II molecules are transported from ER to Golgi complex and accumulate within lysosome-related internal membrane vesicles or membrane sheets as intracellular compartments known as MHC class II–rich compartments (MIICs). Extracellular soluble and particulate antigens engulfed by iDCs are subjected to partial proteolysis and targeted to MIISCs (Inaba et al., 1997) where they are loaded on to MHC class II molecules and trafficked to the cell surface (Kleijmeer et al., 1995; Nijman et al., 1995). The MHC-II transactivator CIITA has been found to act as a master-regulator of MHC-II gene expression (Muhlethaler-Mottet et al., 1997). In iDCs, class II molecules have a short half-life (about 10 hours) and are rapidly degraded and internalized. Conversely, maturation induced stimuli will cause a burst of class II synthesis and translocation of the MHC class II-peptide complexes to the cell surface with several fold increase in their surface expression of MHC class II molecules where they remain stable for days (half- life increases to over 100 hours) and are available for recognition by $CD4^+$ T cells (Cella et al., 1997a). The major contributor for the build-up of MHC class II molecules at the mDCs cell surface is the down-regulation of MHC class II degradation and internalization. This down-regulation guarantees that the complexes created during maturation process will remain on the cell surface for prolonged periods, allowing mature DCs to display antigens for a sufficient time. Although there is increase in the cell surface MHC class II expression, MHC class II mRNA de novo biosynthesis is turned off

during DC maturation and this is due to a remarkably rapid reduction in the synthesis of class II transactivator (CIITA) mRNA (Landmann et al., 2001).

1.6 Activation of T cells by antigen bearing mature DCs

When a naïve T cell has recognised, via its T cell receptor (TCR), a specific antigen presented by the DC, a specialized area of contact between T cell and DC called the immunological synapse is formed. This synapse consists of clusters of TCRs with MHC-peptides surrounded by a ring of adhesion molecules (Grakoui et al., 1999). Signals through the TCR render the T cell fully activated. It will undergo several rounds of proliferation resulting in its distinct clonal expansion and functional differentiation into effector T cells of either $CD4^+$ T helper cells or $CD8^+$ cytotoxic T cells (CTLs) depending on the type of MHC molecules presented on DCs being either MHC class II or MHC class I molecules respectively (first signal). Additionally, T cells require a second signal for full activation. This second signal comes from co-stimulatory receptors expressed by DCs (Dhodapkar et al., 2000; Mellman and Steinman, 2001). The experimental study of DC induced T cell activation requires the use of transgenic systems and these are briefly described in Box 1.

1.6.1 Role for DC co-stimulatory receptors in T cell activation

Dendritic cell maturation is characterized by up-regulation in the expression of co-stimulatory molecules such as CD40, CD80, and CD86 (Hart, 1997). These molecules do not initiate antigen-specific T cell responses but rather help in their generation and amplification by providing the second signal that is needed for the complete activation of T cells. Furthermore, without co-stimulatory molecules, antigen-specific T cell stimulation will lead to T cell anergy or cell death (Guinan et al., 1994). Immature DCs present self-antigens to T cells and in the absence of co-stimulatory molecules results in their deletion or inactivation thus preventing an autoimmune response (Bachmann et al., 1999a; Bour-Jordan and Bluestone, 2009). Conversely, if self-antigens are presented on DCs expressing high co-stimulatory

signals, this may lead to the inappropriate activation of auto-reactive T cells resulting in an autoimmune response (Fife and Bluestone, 2008).

Co-stimulatory molecules expressed on DCs engage with their counterpart molecules on T cells and one of the dominant co-stimulatory pathways is the CD80/CD86 on DCs with its ligand CD28 on T cells (Fife and Bluestone, 2008; June et al., 1994). Binding of CD80/CD86 to CD28 will transmit co-stimulatory signals into T cells that lead to amplification of the signalling processes within T cells (Viola et al., 1999), significantly lowering the TCR activation threshold and allowing naive T cells to be readily activated (Lenschow et al., 1996).

1.6.2 Role for DC cytokines in T cell activation and differentiation

Functional differentiation of naïve T cells into effector T cells is highly influenced by the cytokines secreted by the DCs themselves (third signal) (Banchereau et al., 2000). Additionally, the cytokine environment has an impact on DC functions that can be altered by anti-inflammatory cytokines such as IL-10 (Buelens et al., 1995; De Smedt et al., 1997) and TGF-β (Takeuchi et al., 1997) or with steroids (Piemonti et al., 1999) or prostaglandin E2 (Kalinski et al., 1998). Cytokines are secreted small proteins that are considered to be mediators of immune and inflammatory reactions. They may act in an autocrine mode influencing the biology of the cell that releases them, or in a paracrine fashion, affecting the biology of adjacent cells. Some cytokines are stable enough to act in an endocrine mode, modulating the biology of very distant cells from the site of cytokine release (Wang et al., 2004). Dendritic cell derived cytokines can govern the type of T cell response that develops depending on the type of cytokine they produce. For example IFN-γ generates TH1 cells, IL-4 generates TH2 cells or IL-10 generates regulatory T cells (Furio et al., 2009; Iwasaki and Kelsall, 1999). The cytokines secreted by DCs in response to stimulation through different pattern recognition receptors (PRR) include: IL-1β, IL-6, IL-8, IL-10, IL-12, and TNF-α (Dodge et al., 2003; Reis e Sousa et al., 1999; Scanga

et al., 2002; Schakel et al., 2002; Sporri and Reis e Sousa, 2005; Trinchieri, 1995). The wide range of pathogen recognition by DCs, due to their various PRRs expression, result in the variation of cytokines produced thus linking the type of pathogen encountered to the type of T cell response elicited.

Box 1: Measurement of antigen-specific T cell responses

The identification and isolation of monoclonal $CD8^+$ T cells of known antigen-specificity directly from humans or animals is difficult due to the low copies of T cells expressing the same TCR. In order to isolate a specific T cell clone, T cells from an immunised individual are subjected to repeated rounds of re-stimulation with the same antigen. Cloning of single antigen-specific T cells can be accomplished by the re-culturing of proliferating cells in serial dilutions of cultures. To enable the comprehensive study of immune responses of T cells bearing known TCRs that recognise specific antigens, a number of transgenic mice have been generated (Hogquist et al., 1994; Kurts et al., 1997b; Teague et al., 2004). These TCR transgenic mice have T cells that carry a single type of TCR with known antigen-specificity (Hogquist et al., 1994). For example, the generation of DO.11.10 , OT-II and OT-I transgenic mice allows us to study the activation and proliferation of naive T cell responses to antigens derived from ovalbumin in the context of MHC class II (DO.11.10 and OT-II) and MHC class I (OT-I). However, the transgenic T cell receptors can mix up peptides with native receptors to produce hybrid molecules with specificity against self-antigens. The F5 TCR transgenic mouse is another TCR transgenic mouse model that has been extremely useful in studying antigen-specific $CD8^+$ T cell responses (Mamalaki et al., 1993). The generation of a transgenic mouse expressing on most T cells a transgenic T cell receptor (termed F5) specific for a peptide (termed NP68, aa 366-374) derived from the nucleoprotein (NP) of influenza A virus (Mamalaki et al., 1993) allows for robust quantification of T cell responses in the context of defined doses of the viral antigenic peptide, NP68. $CD8^+$ T cells have been shown to be important in viral infection (Butz and Bevan, 1998). Cytotoxic $CD8^+$ T cells are able to directly kill virus infected cells by delivering granzymes to the target cells via pores formed by the insertion of perforin through the cytoplasmic membrane of target cells. Furthermore, $CD8^+$ T cells can mediate target cell apoptosis by engaging Fas (CD95) molecules on the target cells with FasL (CD178) expressed on the CD8 T cell surface (Parkin and Cohen, 2001).

1.7 Key signalling pathways involved in DC maturation

Intracellular signalling cascades are essential for the transduction of DC receptor (e.g. PRRs, CD40 and cytokine receptors) engagement to DC maturation. The key signalling pathways involved in DC maturation are the nuclear factor kappa B (NF-κB) and the mitogen activated protein kinases (MAPKs). A major trigger for these signalling pathways is through the TLRs which are prototype PRRs expressed in

sentinel cells such as macrophages and dendritic cells. To-date, 13 different types of PRRs has been recognised. They can recognize pathogen-associated molecular patterns (PAMPs) from microorganisms or danger-associated molecular patterns (DAMPs) from damaged tissue (Akira et al., 2001). The different TLRs and their respective ligands are shown in **Table 1.1**.

Receptor	Ligand	Ligand Source
TLR1	Triacyl lipopeptides	Bacteria and mycobacteria
TLR2	Lipoprotein Peptidoglycan	Bacteria Mycobacteria Fungi
TLR3	Double-stranded DNA	Viruses
TLR4	Lipopolysaccharide Taxol	Bacteria (Gram negative) Plants
TLR5	Flagellin	Bacteria
TLR6	Diacyl lipopeptides Zymosan	Mycoplasma Fungi
TLR7	Imidazoquinoline Loxobrine Single-stranded RNA	Synthetic compound Synthetic compound Viruses
TLR8	Single-stranded RNA	Viruses
TLR9	CpG-containing DNA	Bacteria and viruses

Table 1.1 Summary of Toll like receptor classes and ligands. Adapted from Akira & Takeda 2004.

DC maturation is initiated when a TLR is engaged by its ligand, for example binding of TLR4 by the bacterial cell wall component, lipopolysaccharide (LPS). TLR4 is a type I transmembrane protein with an extracellular domain consisting of a leucine-rich repeat (LRR) domain responsible for the recognition of pathogens, and a

cytoplasmic domain, required for initiating intracellular signalling, homologous to the cytoplasmic domain of the interleukin (IL)-1 receptor (TIR) (Medzhitov et al., 1997).

Ligation of a TLR4 promotes its dimerisation and results in the recruitment of the cytosolic adaptor molecule (MyD88) which contains two domains: a C-terminal Toll homology domain that interacts with the Toll homology domain of the receptor, and an N-terminal death domain (DD), however, the efficient triggering of TLR4 response requires the expression of the secreted protein MD-2 (Re and Strominger, 2002). MyD88 also has an intermediate domain (ID) that is crucial in TLR signalling as it interacts with IL-1R associated kinase (IRAKs) (Arancibia et al., 2007). Upon ligand binding to TLR, IRAK is activated by phosphorylation and this subsequently causes its dissociation from the receptor complex and association with tumor necrosis factor (TNF) receptor-activated factor 6 (TRAF6). This event results in activation of the Rel family transcription factor NF-κB and MAPK family (Akira et al., 2001).

Beside the TLR family, DC maturation can be induced by engagement of certain cell receptors with their ligands like co-stimulatory receptor CD40 with CD40 ligand (van Kooten and Banchereau, 2000) and cytokine receptors with interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) (Bernhard et al., 2000). Signal transduction downstream of these receptors shows a complex picture involving different mediators and pathways. These pathways include MAPKs, NF-κB and protein tyrosine kinases (Grammer et al., 1998; Purkerson and Parker, 1998; Rothe et al., 1995).

1.7.1 The NF-κB pathway

NF-κB is a major regulator of the innate and adaptive immune response, cell proliferation, and survival. It is an inducible transcription factor responding to

inflammation, infection and stress stimuli and rapidly reprograms gene expression through activation of an exceptionally large number of genes (Rothwarf and Karin, 1999). Mature DCs express high levels of the NF-κB family of transcription factors (Ouaaz et al., 2002). The NF-κB family members are formed by combinations of five distinct DNA binding subunits, p65/RelA, RelB, c-Rel, p50, and p52 and exist as homo- or heterodimeric complexes. RelA, RelB and c-Rel are synthesized in their mature forms and contain a transactivation domain that interacts with the transcriptional apparatus. Both p50 and p52 lack a transactivation domain although they contain a DNA binding domain; they are synthesized in precursor forms that are proteolysed by the proteasome resulting in the production of mature proteins (Ghosh et al., 1998). In unstimulated cells, NF-κB is found in an inactive form sequestered in the cytoplasm and bound to inhibitory IκB proteins. When cells are stimulated, this leads to rapid phosphorylation, ubiquitination, and eventually proteolytic degradation of IκBα thereby freeing NF-κB. NF-κB then translocates to the nucleus and drives gene expression, including that of IκBα, which facilitates the termination of the transcriptional response by binding and retaining the NF-κB dimers in the cytoplasm genes (Rothwarf and Karin, 1999) as shown in **Figure 1.3**. Phosphorylation of IκBα is induced by the multi-subunit IκB kinase (IKK) which has two catalytic subunits, IKKα and IKKβ and a regulatory subunit IKKγ (NEMO) which is proposed to serve as a recognition site for upstream activators (Rothwarf et al., 1998). Binding of TLR with its ligand will induce IKK activity and thereby activate NF-κB mediated gene transcription. NF-κB targets include the stress-response genes cyclooxygenase 2 (COX 2) and inducible nitric oxide synthase (iNOS), the detoxification enzyme superoxide dismutase (SOD), the apoptotic regulators p53, Bcl-xL and FasL, and proteins involved in antigen presentation and receptors like MHC class II and CD86 (Pahl, 1999). It has been shown that inhibiting NF-κB translocation to the nucleus decreases the up-regulation, by LPS, of MHC class II, CD80 and CD86 (Rescigno et al., 1998). NF-κB has also been implicated in DC

development, maturation, and survival through transcription of a number of different genes, including those coding for chemokines (IL-8), cytokines (IL-1, IL-2, IL-12, and TNF-α), and adhesion molecules (endothelial leukocyte adhesion molecule, vascular cell adhesion molecule, and intercellular adhesion molecule) (Caamano and Hunter, 2002).

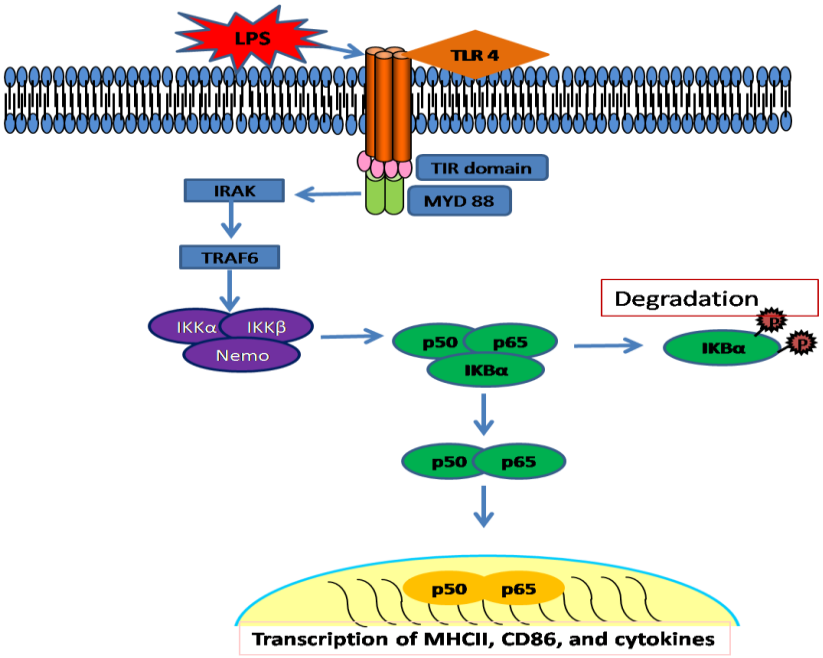


Figure 1.3 Signalling cascades leads to NF-κB activation.

1.7.2 The MAPK pathways

The MAPKs represent vital intracellular signalling pathways that can be activated by an enormous array of stimuli leading to regulation of a variety of cellular processes including cell differentiation, proliferation, and apoptosis (Chang and Karin, 2001). Classical MAPKs pathways consist of at least three-modular cascades that involve activation by phosphorylation of downstream kinases by upstream kinases. Engagement of cell receptors with their ligands causes recruitment of cytoplasmic domain receptors, through multi-protein complexes, with the MAPK kinase kinases (MAPKKKs or MAP3Ks) leading to their activation and phosphorylation, these

phosphorylated MAPKKKs subsequently phosphorylate MAPK kinases (MAPKKs or MAP2Ks or MEKs), which in turn will phosphorylate MAPKs. Different MAPKKKs exist and have large regulatory domains that interact with upstream regulators. MAPKKs typically have smaller regulatory domains and are activated by dual phosphorylation of serine and threonine residues within the activation loop of the catalytic domain. They show great specificity for their cognate MAPK but are regulated by multiple MAPKKKs (Qi and Elion, 2005). The sequential activation of the individual components of the MAPK cascade enables the final MAPK to be phosphorylated and thus modify the activity of its downstream effector molecules to generate a specific intracellular response (Lewis et al., 1998). In DCs, MAPKs pathways regulate their maturation and modulate the type of immune response in concert with NF-κB and other pathways (Breckpot and Escors, 2009). Three groups of classical MAPKs have been identified: the extracellular signal-regulated protein kinases (ERK) (Boulton et al., 1991), the c-Jun N-terminal kinases (JNK) (Kyriakis et al., 1994) and the p38 stress-activated protein kinases (p38MAPK) (Lee et al., 1994). They are structurally related and their catalytic domain is blocked by a conserved activation loop. When they are activated by phosphorylation, the loop unblocks the catalytic site leading to phosphorylation of specific targets. All the MAPKs contain a Thr-X-Tyr (TXY) motif within their activation loop. The phosphorylation of both threonine and tyrosine within the activation loop is essential and sufficient for their activation (Zhang and Dong, 2007). They are activated by specific MAPKKs: MEK1/2 for ERK1/2, MEK3/6 for the p38MAPK and MEK4/7 for the JNKs (JNKK1/2) (Chang and Karin, 2001).

MAPKs activation, under physiological conditions, is often transient. The actual levels of MAPKs do not change throughout the course of stimulation; therefore, dephosphorylation by phosphatases would seem to play a major role in the down-regulation of MAPK activity. Mitogen-activated protein kinase phosphatases (MKPs) are dual protein phosphatases that can dephosphorylate both the phosphotyrosine

and phosphothreonine residues on activated MAPKs (Liu et al., 2007). Structurally, all MKPs have a highly conserved C-terminal catalytic domain and a less conserved N-terminal region that engages the cognate MAPKs (Liu et al., 2007). MAPK phosphatase-1 (MKP-1) is the typical member of the MKP family and has activity, along with MKP5, for p38MAPK (Franklin and Kraft, 1997; Sun et al., 1993; Tanoue et al., 1999). For ERK1/2 the key MKPs are MKP2, MKP3 and MKP4 (Guan and Butch, 1995; Muda et al., 1996; Muda et al., 1997), whereas for JNK it is MKP7 (Matsuguchi et al., 2001). However, it is pertinent to note that a single MKP can simultaneously dephosphorylate multiple MAPKs.

1.7.2.1 Extracellular signal-regulated protein kinases 1/2 (ERK1/2)

The extracellular signal-related kinase (ERK) family consists of: ***A.*** Classical MAPK-ERK which include p44mapk/erk1 (ERK1) and p42mapk/erk2 (ERK2) and are closely related to each other (90% identity) and activated in response to growth factors through serine/threonine phosphorylation (Boulton et al., 1991), and ***B.*** the large MAPKs such as ERK3, ERK5, ERK7, and ERK8 that consist of both a kinase domain and a C-terminal domain and range in size from 60 to greater than 100 kDa (Abe et al., 1996; Kuo et al., 2004; Lechner et al., 1996). Both ERK1 and ERK2 are activated by upstream MAPKKs (MEK1/2). Stimulation can be induced by engagement of a variety of pathogenic or mitogenic stimuli including LPS, growth factors, cytokines, and phorbol esters, with TLRs and G protein coupled receptors (An et al., 2002; Qi and Elion, 2005; Rescigno et al., 1998). Activated MEK1/2 phosphorylates threonine and tyrosine residues in the Thr–Glu–Tyr (TEY) sequence of ERK1/2, resulting in the activation of ERK1/2. Activated ERK1/2 phosphorylates many substrates including transcription factors, such as ETS domain-containing protein ELK-1 and cAMP response element-binding protein (CREB) and protein kinases, such as ribosomal S6 kinase (RSK). These induce transcription of immediate early genes, such as c-Fos (Agrawal et al., 2003; Yang et al., 1998a) as shown in **Figure 1.4**.

In DCs, the ERK signalling pathway negatively regulates maturation and IL-12 production upon LPS stimulation but positively regulates inflammatory cytokine production like TNF-α, IL-1β, IL-6, or IL-8 (Nakahara et al., 2006; Yanagawa et al., 2002). Additionally, the ERK signal-transduction pathway negatively regulates NF-κB DNA- binding and IκBα levels on TNF-α –induced maturation with enhancement of allogenic T cell response elicited by TNF-α matured dendritic cells (Puig-Kroger et al., 2001)

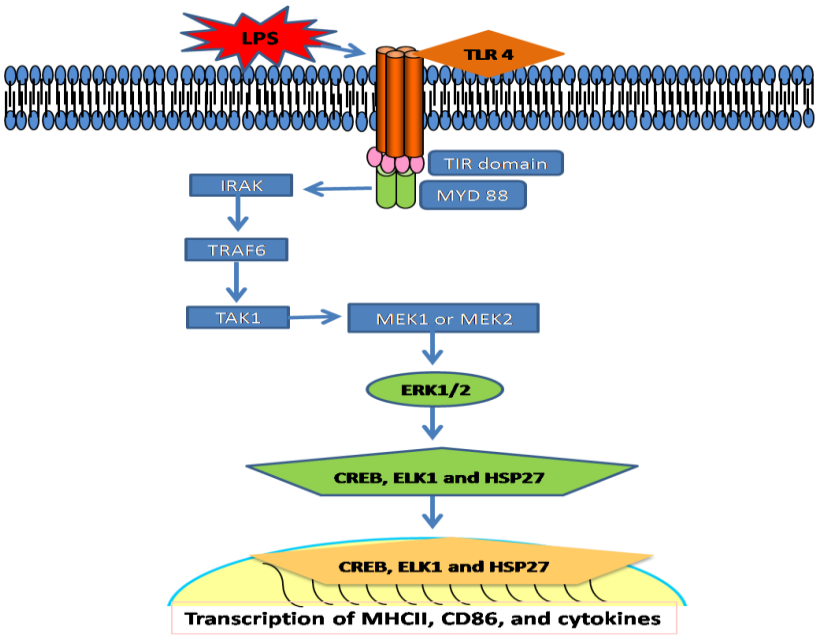


Figure 1.4 Signalling cascades leads to ERK1/2 activation.

1.7.2.2 c-Jun N-terminal kinases (JNKs)

c-Jun N-terminal kinases are serine threonine stress-activated protein kinases that can be induced in response to a variety of extracellular stresses such as bacterial endotoxin, inflammatory cytokines, UV radiation, osmotic shock, and hypoxia (Kyriakis et al., 1994). Three JNK genes (*Jnk1*, *Jnk2* and *Jnk3*) are found in mammals; the *Jnk1* and *Jnk2* genes are expressed ubiquitously, while the *jnk3* gene has a more

limited pattern of expression and is largely restricted to the brain, heart, and testis. They are activated by the MAPK kinases MEK4 and MEK7, via dual phosphorylation, on a specific Tyr residue by MEK4 and a specific Thr residue by MEK7 in a typical Thr–Pro–Tyr (TPY) motif within their "activation/phosphorylation loop" sequences (Davis, 2000). They have the ability to bind and phosphorylate the N-terminal transactivation domain of the transcription factor c-Jun and increase its transcriptional activity (Kyriakis et al., 1994). c-Jun is a component of the signal-responsive transcription complex activated protein-1 (AP-1), which is involved in regulation of gene expression (Leppa and Bohmann, 1999). Additionally, JNKs can also phosphorylate a variety of transcription factors such as ATF2, NFAT, Elk-1 and p53 (Buschmann et al., 2001; Chow et al., 1997; Gupta et al., 1995; Yang et al., 1998b) (**Figure 1.5**).

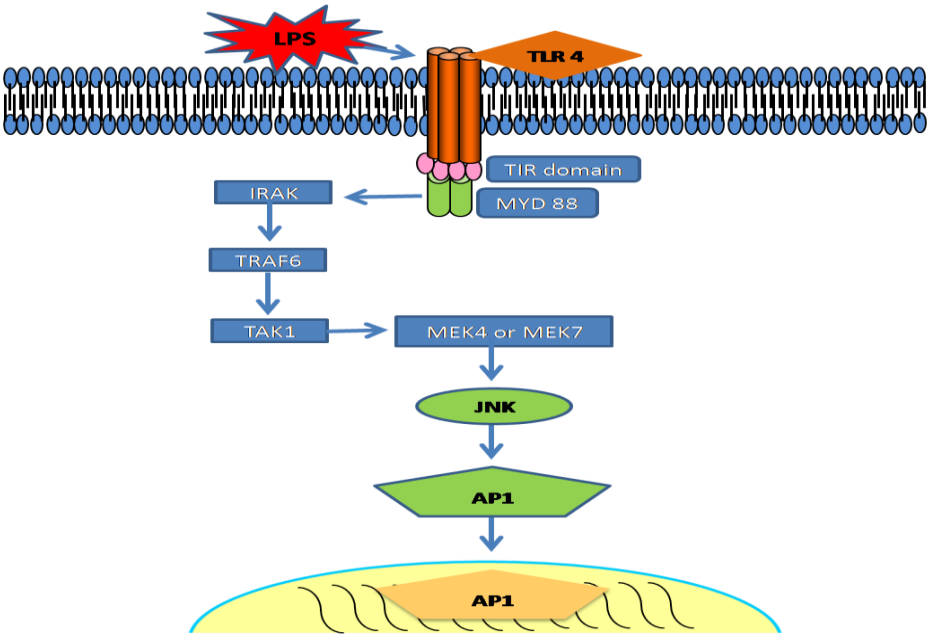


Figure 1.5 Signalling cascades leads JNK activation.

JNKs can activate apoptotic signalling by directly modulating the activities of mitochondrial pro- and anti-apoptotic proteins like Bcl-2 or Bcl-X through phosphorylation or through up-regulation of pro-apoptotic genes by enabling

transactivation of a wide range of transcription factors such as JunD, activating transcription factor 2 and 3 (ATF2, ATF3), Elk-1, Elk-3, p53, Retinoid X receptor alpha and beta (RXRα, RARβ), androgen receptor (AR), Nuclear factor of activated T cells 4 (NFAT4), Heat shock factor protein 1 (HSF-1) and c-Myc (Dhanasekaran and Reddy, 2008; Johnson and Nakamura, 2007). In DCs, LPS is found to induce JNK activation by phosphorylation mainly by MEK7 (Yoshizawa et al., 2008) and a specific inhibitor of JNK, SP600125, inhibited the LPS-induced up-regulation of co-stimulatory molecules CD80 and CD86, but augmented the up-regulation of MHC class II (Handley et al., 2007; Nakahara et al., 2004).

1.7.2.3 The p38MAPK pathway

The p38MAPK participates in many aspects of immune cell functional responses, including respiratory burst activity, chemotaxis, cell differentiation, granular exocytosis, adherence, and apoptosis (Ono and Han, 2000; Roux and Blenis, 2004). Activation of p38MAPK is critical for DC maturation and secretion of pro-inflammatory cytokines; and it is constitutively activated at a low level in mature DCs (Fukao et al., 2000).

Four splice variants of the p38MAPK family have been identified: α (XMpk2/CSBP), β (Beta22), γ (SAPK3/ERK6), and δ (SAPK4). While p38γ and p38δ are differentially expressed depending on tissue type, p38α and p38β are ubiquitously expressed. The p38α enzyme is the best characterized and is expressed in most cell types including DCs (Zarubin and Han, 2005). In general p38MAPK can be activated by various stimuli, such as LPS, proinflammatory cytokines (e.g. IL-1β and TNF-α), and various environmental stresses (osmotic stress, heat, and UV irradiation) (Arrighi et al., 2001; Raingeaud et al., 1995). Activation of p38MAPK pathway can enhance protein expression by its direct effect on gene transcription, by the activation of specific transcription factors, or through post-transcriptional regulation (mRNA

stability, mRNA processing, protein stability, nuclear export, and translation) (Raingeaud et al., 1996).

All p38MAPKs can be phosphorylated specifically on a Thr–Gly–Tyr (TGY) dual phosphorylation motif by upstream MEK3 and MEK6 kinases (Moriguchi et al., 1996). However, upstream phosphorylation of the MEKs/p38MAPK signalling pathways is less specific, which may explain why diverse stimuli can activate the p38MAPK pathway. Several MK kinases (MAP2Ks) have been reported to lead to p38MAPK activation such as: MTK1, MLK2/MST (Cuenda and Dorow, 1998; Hirai et al., 1997; Takekawa et al., 1997), MLK3/PTK/SPRK (Tibbles et al., 1996), ASK1/MAPKKK5 (Ichijo et al., 1997), DLK/MUK/ZPK (Fan et al., 1996), and TAK1 (Moriguchi et al., 1996). Increased activity of these MAP2Ks leads to activation of both the p38MAPK and JNK pathways, explaining why p38MAPK and JNK are often co-activated. However, specific activation of either p38MAPK or JNK has also been observed, indicating selectivity in this pathway at this level (Ogura and Kitamura, 1998). In addition to the downstream differential effects of the MAP2Ks, different upstream signals can be mediated by different MAK3Ks. For example, MTK1 may only mediate stress signals but not those from cytokines. This is evident by the observation that the dominant negative MTK1 mutant can only inhibit the activation of the p38MAPK pathway by environmental stress (osmotic shock, UV, and anisomycin), but not by the cytokine TNF-α (Takekawa et al., 1997). The downstream targets of p38MAPKs vary from kinases such as MK2 and MK3 (MAPKAP kinases) to transcription factors such as CREB, ATF1, ATF2, p53, c/EBPβ and NFAT (Zarubin and Han, 2005) (**Figure 1.6**).

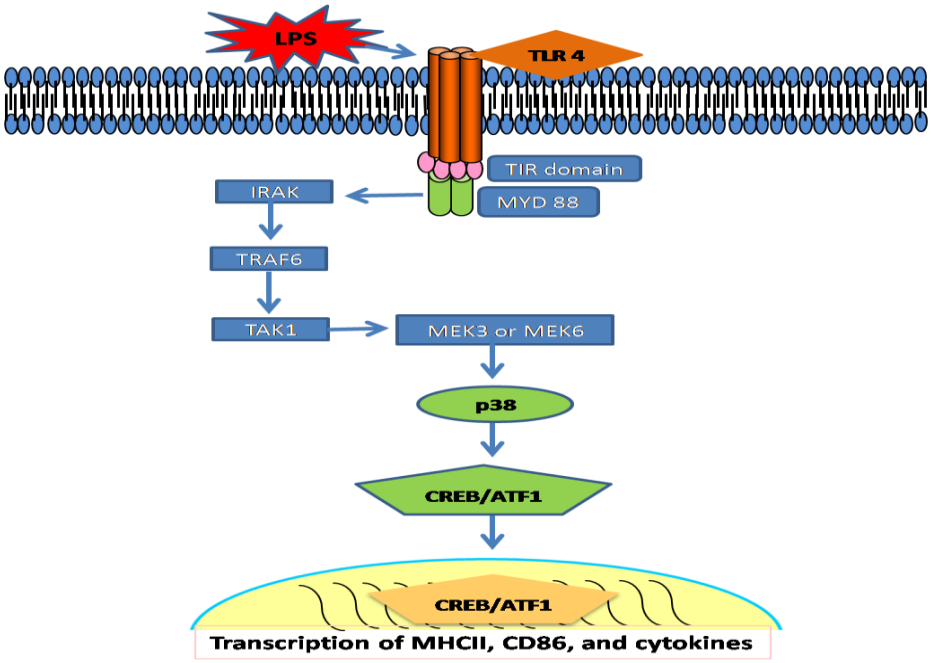


Figure 1.6 Signalling cascades leads to p38MAPK activation.

1.7.2.4 The transcription factors CREB/ATF1 are targets of MAPKs

The regulation of gene transcription is important for both tissue specific-gene expression and regulation of gene activity in response to specific stimuli (Latchman, 1997). Transcription factors (TFs) have crucial roles in mediating gene expression and hence in almost controlling all biological processes. TFs contain a basic domain that binds specific DNA sequence (DBDs) which influence the rate of transcription (Jakoby et al., 2002). CREB and the closely related protein ATF1 constitute a subfamily of bZIP transcription factors that binds to its DNA target sequence as a dimer and play critical roles in the regulation of gene expression as well as cell cycle, metabolism, growth, and survival (Mayr and Montminy, 2001). CREB and ATF1 represent the major transcriptional effectors of MAPKs (particularly p38MAPK but also ERK1/2). These transcription factors bind to DNA sequences termed cAMP responsive elements (CRE) and drive gene expression.

CREB activation occurs through phosphorylation of its Ser133 and Ser142 residues within the 60-amino-acid kinase-inducible domain (KID). ATF1 is structurally and functionally related to CREB and is activated by phosphorylation at Ser63 and

Ser133 (Shaywitz and Greenberg, 1999). ATF1 mediates its responses as homodimers or as heterodimers with CREB. ATF1/CREB heterodimers have a longer half-life compared with ATF1/ATF1 homodimers, when bound to the cAMP response elements (CRE) (both approximately several minutes), while CREB/CREB homodimers have the longest half-life (10–20 min). Therefore, CREB appears to be responsible for the bulk of essential functions carried out by CREB/ATF1 family members. The duration of CREB phosphorylation is of significance in determining whether transcription occurs or not (Wu et al., 2001).

Gene transcription ability of CREB/ATF1 depends on its phosphorylation state, which, in turn, regulates its recruitment to the versatile co-activators like CREB-binding protein (CBP) or p300, both responsible for trans-activation of the cell transcriptional machinery. CREB/ATF1 may compete with other transcription factors such as NF-κB for recruitment and binding of CBP (Gerritsen et al., 1997; Katoh et al., 2001; Vo and Goodman, 2001). Genes that are CREB-responsive include the proinflammatory cytokine TNF-α (Roach et al., 2005) and cyclooxygenase 2 (COX2) (Eliopoulos et al., 2002), anti-inflammatory cytokines such as IL-10 (Platzer et al., 1999) and the pro-survival gene, *bcl-2* (Chao and Korsmeyer, 1998).

In DCs, phosphorylation of CREB is known to be associated with up-regulation of co-stimulatory molecules (CD80 and CD86) (Ardeshna et al., 2000) and increased secretion of IL-10 (Alvarez et al., 2009).

CREB is inactivated by phosphatases like protein phosphatase 1 (PP1) and protein phosphatase A2 (PPA2) through dephosphorylation at Ser-133 (Hagiwara et al., 1992; Vo and Goodman, 2001; Xing et al., 1998).

1.8 Redox homeostasis and immune cell function

Redox homeostasis can be generally defined as a relatively stable state of equilibrium or a tendency towards such a state between the diverse but mutually dependent oxidative and antioxidant elements or groups of elements of an organism (Buettner, 1993). Redox homeostasis is important for a variety of cellular functions such as proliferation, apoptosis, and intracellular signalling pathways including MAPK signalling (Go et al., 2004).

Disturbance in redox homeostasis leads to a condition known as oxidative stress which can be defined as a disorder in the pro-oxidant–/antioxidant balance in favour of the former, leading to potential injury. Disturbances in this normal redox state can cause toxic effects resulting in damage to components of the cell, including DNA, proteins, and lipids (Trachootham et al., 2008).

Oxidative damage is characterised as the biomolecular impairment caused by the attack of reactive species upon the constituents of living organisms.

Oxidative stress can have deleterious effects on cell function including immune cell function. It has been widely recognized that oxidative stress is involved in the aggravation of immune disorders such as pulmonary injury, inflammation and emphysema (associated with chronic obstructive pulmonary disorders, COPD) (Matthay and Zimmerman, 2005).

Oxidative stress leads to a very wide spectrum of metabolic, genetic, and cellular responses. Most oxidative stress conditions that cells might come across may stimulate cell growth, modulate gene expression, or may cause a transient adaptive response and protective temporary growth-arrest. In the most extreme outcome, which involves direct cell destruction, necrosis occurs (Davies, 2000).

A large number of pro-oxidants are generated within the cell and the organism as well as from exogenous sources as shown in Box 2 (Nathan, 2003). These pro-oxidants include reactive oxygen species (ROS) which comprises superoxide,

hydrogen peroxide, singlet oxygen, ozone, hypohalous acids, and organic peroxides, and reactive nitrogen species (RNS) which comprises nitric oxide ($NO^{\bullet}$) and $NO_2^{\bullet}$; hydrogen sulphide (H_2S) and its anion HS^{-}.

Reactive oxygen species are free radicals that are extremely reactive owing to the presence of unpaired electron/s in their highest atomic or molecular orbitals. Reactive oxygen species are generated as by-products of aerobic metabolism or exposure to various natural and synthetic toxicants (Davies, 2000). Furthermore, ROS can be induced by the accumulation of hemin, lipid aldehydes, and arsenic (Hsieh et al., 2006; MacKenzie et al., 2008; Reichard et al., 2007; Tjalkens et al., 1998).

Transient, low level, and controlled production of ROS is part of normal cell physiology and plays an essential role in signal transduction cascades, protein ubiquitination and degradation, as well as cytoskeleton arrangement (Yan et al., 2008). However, excessive or prolonged ROS could cause lipid peroxidation, protein amino acid side-chain oxidation, and DNA single- and double-strand breaks (D'Autreaux and Toledano, 2007). These different effects of ROS influence cell cycle progression which is dependent upon the amount and duration of ROS exposure (Boonstra and Post, 2004). ROS are found to be related to a wide variety of human disorders, such as chronic inflammation, age-related diseases, and cancers.

ROS can modulate immune responses through affecting many physiological processes in different types of cells. For example in macrophages, ROS contributes to the expression of inflammatory mediators by acting as second messengers and are necessary for the induction of the respiratory burst which is essential for macrophage and neutrophil pathogen destruction (Forman and Torres, 2002; Gwinn and Vallyathan, 2006; Schaur et al., 1994). Additionally, ROS levels were increased after LPS challenge in peritoneal macrophages and lymphocytes in different models of septic shock causing increase in adherence and phagocytosis and a decrease in chemotaxis (Victor et al., 2004).

Box 2: Sources of ROS and mediators of their catabolism (Adopted from Nathan 2003)

Exogenous sources of ROS	**Endogenous sources of ROS**
•Smoke •Air pollutants •Ultraviolet radiation •γ-irradiation •Several drugs	•NADPH oxidases •Mitochondria •ER flavoenzyme ERO1 •Xanthine oxidase •Lipoxygenases •Cyclooxygenases •Cytochrome P450 enzymes •Flavin-dependent demethylase •Polyamine and amino acid oxidases •Nitric oxide synthases •Free iron or copper ions •Haem groups •Metal storage proteins

Catabolism by antioxidant systems	**Catabolism by small molecules that react with ROS non-enzymatically**
•Superoxide dismutases •Catalases •Glutathione peroxidases •Glutathione reductase •Thioredoxins •Thioredoxin reductases •Methionine sulphoxide reductases •Peroxiredoxins or peroxynitrite reductases	•Ascorbate •Pyruvate •α-ketoglutarate •Oxaloacetate

Evidence has shown that the intracellular redox status and ROS can serve as signalling mediators to initiate multiple signalling pathways in DCs. This can result in co-stimulatory molecule expression (MHC class II and CD86) up-regulation which have significant effects on DC functions with subsequent impact on the type of T cell immune response elicited (Kantengwa et al., 2003; Kim et al., 2007; Williams et al., 2008). Furthermore, ROS may induce DC chemokines and cytokines production through NF-κB and MAPKs pathways so that they can act as a "danger" signal from the inflammatory micro-environment leading to DC activation, and hence an antigen-specific immune response (Rutault et al., 1999; Yan et al., 2008). Previous studies have shown that modulating redox balance in DCs leads to reduction in IL-

12 and TNF-α cytokine production (Yamada et al., 2006) and decrease in antigen-specific T cell proliferation and IFN-γ synthesis (Tse et al., 2007).

1.9 Influence of redox on signalling pathways

Several reports have shown, in many cell types, that an altered redox status results in the activation of several intracellular signalling pathways like MAPKs and NF-κB, suggesting a role for ROS as second messengers (Gaitanaki et al., 2003; Schaeffer et al., 2003; Wang et al., 1998). ROS may have direct protein target interactions with these signalling pathways wherein exposure of these molecules to an altered redox state alters the function of the target protein. Some recent evidence supports this notion and suggests that both certain protein-protein interactions and enzymatic functions might be regulated by cellular ROS levels (Finkel, 2000). ROS was found to induce the phosphorylation of p38MAPK, ERK, and JNK members of MAPK family and inhibition protein phosphatases (Lee and Esselman, 2002).

1.10 Cellular defence against reactive oxygen species

At the cellular level, an extensive defence system against ROS has evolved, this system includes: small thiol-rich molecules such as glutathione and metallothioneins that can directly inactivate ROS; and enzymes such as heme-oxygenases, SOD, NAD(P)H:quinone oxidoreductases (NQO), glutathione S-transferases (GST), catalase (CAT) and peroxidases which metabolise ROS into non-reactive metabolites (Chelikani et al., 2004; Halliwell and Gutteridge, 1990; Miao and St Clair, 2009; Ross, 2004). A second line of defence against oxidative stress involves small molecules that react with ROS non-enzymatically and can be recycled or replenished, giving them a ROS-buffering capacity. These include ascorbate, α-tocopherol, and the α-ketoacids of central carbon metabolism, such as pyruvate, α-ketoglutarate and oxaloacetate (O'Donnell-Tormey et al., 1987).

1.10.1 Thiol-containing cellular antioxidants

Glutathione is an important tripeptide (glutamate, cysteine, and glycine) thiol molecule that protects cells against oxidative damage from ROS generated during normal metabolism and xenobiotic exposure, and it also functions as an important substrate in enzyme catalysed detoxification reactions (DeLeve and Kaplowitz, 1991). Glutathione exists in thiol-reduced (GSH) and disulfide-oxidized (GSSG) forms. Under physiological conditions glutathione reductase will rapidly reduce any GSSG to the reduced, thiol form, GSH. More than 98% of intracellular glutathione is GSH. The rest is present within the cell as the oxidized disulfide form (GSSG), as mixed disulfides (mainly GSS-protein) and as thioethers (Kaplowitz et al., 1985). γ-Glutamylcysteine synthetase (γ -GCS) is a rate-limiting enzyme involved in the de novo synthesis of GSH, and is structurally made up of glutamate cysteine ligase, catalytic subunit (GCLc) and glutamate cysteine ligase, regulatory subunit (GCLm) (Jeyapaul and Jaiswal, 2000). The antioxidant capacity of GSH does not only come from its direct ability to react with ROS alone, but also from its function as a substrate for GSH peroxidases (GPX), which catalyse the reduction of hydrogen peroxide (H_2O_2) and lipid hydroperoxides (DeLeve and Kaplowitz, 1991). Additionally antioxidant capacity of GSH can be attributed to GSTs, which constitute a family of enzymes that detoxify xenobiotics by conjugating GSH to a range of electrophilic substrates (Thimmulappa et al., 2002). These enzymatic activities yield GSH disulphide (GSSG), which is reduced back to GSH via a nicotinamide adenine dinucleotide phosphate (NADPH) dependent, GSH reductase-mediated reaction, a cycle that serves to maintain essential redox balance (DeLeve and Kaplowitz, 1991).

Another important thiol-containing molecule is thioredoxin (TRX), a small protein that contains two critical cysteine residues (32 and 35) (Haendeler, 2006). The preservation of cysteine redox state is particularly important for the function of certain enzymes whose catalytic activity is dependent upon the integrity of a

sulphydryl or disulphide moiety. The cysteine thiolate residue (Cys-S) is more readily oxidized by peroxides than is the Cys-sulfhydryl residue (Cys-SH) which serve as the immediate electron donor for the peroxidase function (Rhee et al., 2005). This leaves a reduced or dithiol target protein and a disulfide in the active site of TRX. This disulfide is reduced by TRX reductase (TRXR) using electrons from NADPH (Berndt et al., 2007). The peroxiredoxins (PRX) are a protein family which contain a conserved Cys residue that undergoes a cycle of peroxide-dependent oxidation and thiol-dependent reduction during the catalysis process which is capable of directly reducing peroxides, e.g. hydrogen peroxide and different alkyl hydroperoxides (Rhee et al., 2005).

1.10.2 Enzymes as effectors of redox homeostasis

Among the enzymes that are involved in countering ROS are members of the SOD family that utilise the transition metals copper and zinc (Cu, Zn-SOD present in cytosol Mn-SOD present in mitochondria) at their active sites, and catalyse the dismutation of two superoxide anion radicals ($O2^{\cdot-}$) to H_2O_2 and O_2. The H_2O_2 cytotoxic product of this reaction is subsequently detoxified by GPX, and/or PRX producing H_2O and O_2 (Nordberg and Arner, 2001). Catalases also accelerate the degradation of two molecules of H_2O_2 into water and oxygen (Chelikani et al., 2004). NADP(H)-quinone oxidoreductases (NQO) are flavoenzymes that catalyse the two-electron reduction of quinones to hydroquinones (Vasiliou et al., 2006). Two isoforms of the enzyme exist, NQO1 (molecular mass of 31 kDa) utilizes NAD(P)H, whereas NQO2 (molecular mass of 25 kDa) employs dihydronicotinamide riboside (NRH) as the electron donors. NQO1 also has other cytoprotective properties such as maintenance of the endogenous lipid-soluble antioxidants α-tocopherol and direct scavenging of O_2^- radicals via NAD(P)H- dependent redox reactions (Ross, 2004; Siegel et al., 2004). These enzymes are widely distributed through mammalian tissues with the highest levels being in liver and cardiovascular tissues (Vasiliou et al., 2006).

1.10.3 Small non-protein antioxidant molecules

α-Tocopherol (vitamin E) is a potent lipid-soluble antioxidant that has a primary role in breaking chain reactions involving O_2 and lipid peroxyl free radicals (Yu, 1994). Additionally it can stabilize and activate the redox sensitive transcription factor nuclear factor E2 p45-related factor 2 (Nrf2) (Rahman et al., 2006). Vitamin E plays a central role in disease prevention by scavenging both ROS and RNS (Zingg, 2007). Ascorbic acid (vitamin C) is a hydrophilic antioxidant that has free radical scavenging capacity and is widely distributed in mammalian tissues. In addition to this direct role, vitamin C can restore the antioxidant properties of oxidised vitamin E (Yu, 1994).

Vitamins C and E have been shown to reduce ROS levels in different cell types including neutrophils (Kanno et al., 1995), monocytes (Cachia et al., 1998; Devaraj et al., 1996), and macrophages (Sakamoto et al., 1990). Addition of vitamins C and E to DCs before challenge with stimuli that are known to induce ROS production, resulted in a reduction in ROS levels comparing to untreated DCs (Tan et al., 2005). This effect is more prominent when DCs are incubated with vitamins for longer durations before stimulation. DCs treated with these antioxidant vitamins are found to be resistant to phenotypic and functional changes subsequent to proinflammatory cytokines stimulation (Tan et al., 2005).

Other ROS scavenging molecules include: uric acid (the end product of purine metabolism), ceruloplasmin (a major copper-carrying protein), β-carotene (a metabolic precursor of retinol) and bilirubin (a breakdown product of heme catabolism) (Davies, 2000; Yu, 1994).

In general, the above-mentioned antioxidants demonstrate their scavenging actions by donating an electron to a radical, and thus generating a non-radical species. Their role is considered to be unavoidably suicidal by becoming alternative targets for reactive species in the place of critical macromolecules (Davies, 2000).

Glutathione and detoxifying enzymes expression (both basally and inducibly) are mainly regulated by Nrf2, a redox-responsive transcription factor which has a central role in cellular adaptive and defence systems against oxidative and electorphilic stress (Itoh et al., 1997b; Jaiswal, 2004; McMahon et al., 2001).

1.11 Nrf2

The transcription factor Nrf2 is a member of the cap'n'collar (CNC) basic leucine zipper transcription factors and is essential for the coordinated induction of genes that encodes cytoprotective enzymes or stress-responsive proteins, such as HO-1, NQO1, SODs, GPx, GSTs, GCL, CAT and TRX (Reddy et al., 2007b).

Other members of the CNC family of transcription factors include the Nrf1, Nrf3 and the repressive factors bric-a-brac/tram-track/broad complex (BTB) and CNC homolog 1: Bach1 and Bach2 (Chan et al., 1998; Kobayashi et al., 1999), and are named as such due to structural similarities with the Drosophila protein cap 'n' collar (CNC). Nrf2 contains a C-terminal basic leucine zipper (bZip) structure that facilitates dimerisation and DNA binding (Moi et al., 1994). Nrf1 is less potent than Nrf2 at transactivating gene expression (Zhang et al., 2006), while Nrf3 has been proposed as a negative regulator of Nrf2 by interfering with ARE-mediated transcription (Sankaranarayanan and Jaiswal, 2004).

Nrf2 was first cloned during a screen for nuclear factor erythroid 2 (NF-E2) - regulating proteins in hemin-induced erythroleukemia cells (Moi et al., 1994). It is homologous to another CNC transcription factor nuclear factor erythroid 2 (NF-E2). While NF-E2 regulates globin gene expression in developing erythroid cells (Igarashi et al., 1994), Nrf2 is also highly expressed in non-haemopoietic tissues including heart, muscles, pancreas (Moi et al., 1994), in organs particularly associated with detoxification (liver and kidney), and those that are exposed to the external

environment (skin, lung and digestive tract) (Motohashi et al., 2002). The Nrf2 structure consists of six highly conserved regions named as homology domains (Nrf2-ECH homology) Neh1 to Neh6 as shown in **Figure 1.7** (Itoh et al., 1999).

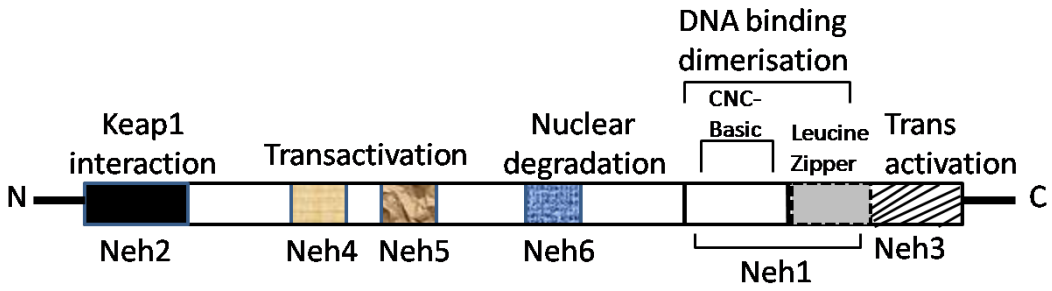


Figure 1.7. Schematic representation for the basic structure of transcription factor Nrf2. Neh1 is the first conserved domain and contains the basic-leucine zipper domain and CNC homology region. The amino and carboxyl termini of the proteins are also highly conserved, and are referred to as Neh2 and Neh3, respectively. Additionally, there are two conserved acidic domains (Neh4 and Neh5) as well as a serine-rich conserved region (Neh6). Adopted from Itoh et al. 1999.

Nrf2 binds to the antioxidant response element (ARE), a DNA motif found within the promoter regions of numerous cytoprotective genes, with high affinity only as a heterodimer with its obligatory partner a small Maf protein (Itoh et al., 1997b). Small Maf proteins lack transactivation domains, and thus the ability of the Nrf2-Maf heterodimer to promote transcription is solely dependent on the transactivation ability of Nrf2 (Motohashi et al., 2002). Amongst members of the CNC family, Nrf2 is the most potent transcriptional activator from the ARE (Kobayashi et al., 1999; Papaiahgari et al., 2006).

Nrf2 requires the recruitment of co-activators that include CBP/p300, co-activator-associated arginine methyltransferase 1 (CARM1), protein arginine N-methyltransferase 1 (PRMT1) and p300/CBP-associated factor (p/CAF) for initiation of Nrf2 related gene transcription (Lin et al., 2006). The cAMP responsive element binding protein (CREB)-binding protein (CBP) has been shown to bind to the

transactivation domains of Nrf2, Neh4 and Neh5, which act synergistically to attain maximum activation of reporter gene expression. Indeed, specific inhibition of CBP significantly reduced Nrf2 activity (Katoh et al., 2001). CBP helps transcription through ***a)*** its intrinsic histone acetyltransferase (HAT) activity, ***b)*** interaction with other proteins possessing HAT activity, and ***c)*** bridging to components of the general transcriptional machinery (Bannister and Kouzarides, 1996; Kalkhoven, 2004). Histone acetylation (as well as DNA methylation) control the access of transcription factors to the promoter regions and thereby affect the pattern of gene transcription. Nucleosomes contain histones in their core, around which DNA is wound into a condensed structure that represses transcription, but that can be selectively unfolded to increase accessibility to general transcription factors and RNA polymerase II, and thus promote gene transcription (Grunstein, 1990; Kuo and Allis, 1998).

Transcriptional repressors Bach1 and Bach2 are basic leucine zipper (bZip) protein and forms heterodimers with one of the small Maf proteins (i.e. MafK, MafF, and MafG) that bind to the Maf recognition element (MARE) and effectively function as repressors of gene transcription over NFE2-related transcription, including suppression of a key gene in heme metabolism, heme oxygenase-1 (HO-1) (Kitamuro et al., 2003; Yoshida et al., 2007).

1.11.1 Kelch-like ECH-associated protein-1 (Keap-1)

Analyses of Nrf2 activity has led to the identification of Nrf2 transcriptional activity suppressor, Kelch-like ECH-Associated Protein 1 (Keap1), which specifically binds to Nrf2 via its evolutionarily conserved amino terminal regulatory domain. In the absence of cellular stress, Nrf2 is tethered within the cytosol by keap1, which binds to Nrf2 via the Neh2 domain (to the seven lysine residues located between the conserved ^{29}DLG^{31} and ^{79}ETGE^{82} motifs) of the transcription factor (Itoh et al.,

1999). This interaction represses cellular Nrf2-dependent transcription activity (Zipper and Mulcahy, 2002).

The Neh2 moiety of Nrf2 is required for the repression activity by Keap1 (Itoh et al., 1999; Xue and Cooley, 1993). Keap1 resides within the cytosol where it interacts with the actin cytoskeleton and, in the absence of chemical/oxidative stress, associates with Nrf2 leading to Nrf2 proteasomal degradation. Structurally, Keap1 contains three major domains; N-terminal BTB domain, an intervening region (IVR) bridge that is rich in cysteine residues and regulates activity of Keap1 and links BTB to double glycine (DGR) domain situated on the C-terminal containing six conserved repeats also known as kelch (**Figure 1.8**) (Itoh et al., 1999; Kang et al., 2004). The BTB domain mediates protein dimerisation which is required for effective Nrf2 sequestration in the cytosol, whereas Kelch repeats have been implicated in binding to the actin cytoskeleton and the formation of multi-protein complexes (Albagli et al., 1995; Robinson and Cooley, 1997). Cullin3 (Cul3), a subunit of the E3 ligase complex, serves as a molecular bridge bringing together substrate adaptor protein (Keap1) and substrate (Nrf2) through Keap1 BTB and intervening-region (IVR) domains. Therefore, Keap1 would participate directly in the regulation of Nrf2 polyubiquitination and subsequent 26S proteasome-mediated degradation (Cullinan et al., 2004; Kobayashi et al., 2004).

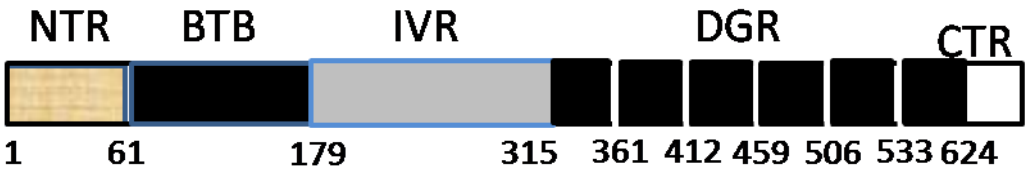


Figure 1.8. Schematic presentation of Keap1. Five domains within Keap1 are demonstrated: NTR, BTB, IVR, DGR, and CTR. Adopted from Kang et al 2004.

1.11.2 Regulation of Nrf2 by Kelch-like ECH-associated protein-1 (Keap-1)

In the absence of chemical and oxidative stress, Nrf2 is sequestered in the cytosol by the repressor protein Keap-1 which directs the transcription factor for ubiquitination and degradation by the 26S proteasome (Zipper and Mulcahy, 2002). Therefore, Nrf2 proteasomal degradation is achieved via its cytosolic interactions with Keap-1 (Kobayashi et al., 2004). Treatment with the organic compound diethyl maleate (DEM) was shown to liberate Nrf2 from Keap-1-mediated sequestration in the cytosol, thus enabling its translocation into the nucleus and binding to the ARE (Itoh et al., 1999). This highlights the pivotal role of keap-1 in the inhibition of Nrf2 activation and nuclear translocation. Under basal conditions, a minimal cytoplasmic pool of Nrf2 is maintained as a result of its rapid degradation by the proteasome pathway, giving Nrf2 a relatively short half-life of approximately < 20 minutes (Katoh et al., 2005; Kobayashi et al., 2004). Studies using proteasome inhibitors that caused the nuclear accumulation of Nrf2 and initiation of Nrf2 target gene upregulation, further emphasised the role of the proteasomal machinery in the regulation of Nrf2 activity (Kobayashi et al., 2004; McMahon et al., 2003). Under resting conditions, Keap-1 presents Nrf2 to the ubiquitin ligase enzyme resulting in its polyubiquitination and rapid degradation via the 26S-proteosome (Ma and He, 2012). However, upon oxidative insult, Keap-1 mediated Nrf2 ubiquitination is inhibited. As a result, Nrf2 accumulates in the cytoplasm allowing it to freely translocate into the nucleus (Kobayashi et al., 2004; Zhang et al., 2004a). Nrf2 regulation by Keap-1 proceeds via a two site recognition mechanism known as the 'Hinge and Latch' model, where hinge refers for ETGE motif and latch for DLG motif (Tong et al., 2006b). When cellular GSH is depleted under oxidative stress conditions, critical cysteine within the BTB and IVR regions of the Keap-1 are modified, resulting in the displacement of Nrf2 from the low affinity DLG binding site; whilst remaining associated to Keap-1 via its high affinity ETGE site (Tong et al.,

2006a). This conformational change in Keap-1 disables it from directing Nrf2 for degradation via the proteasome (Kobayashi et al., 2004). Other Nrf2 molecules then saturate the Keap-1 via binding to the newly available BTB site within the homodimer, subsequently Nrf2-binding capacity of Keap1 is saturated and even diminished due to Keap1 self-ubiquitination allowing any newly synthesised Nrf2 to translocate into the nucleus (Li and Kong, 2009). Nrf2 then associates with small maf protein forming a heterodimer which facilitates its capacity to bind to the ARE regions found in the regulatory domains of its nrf2-regulated genes (Itoh et al., 1997b). The co-activator CBP is then recruited to the Nrf2 heterodimer resulting in its transactivation (Katoh et al., 2001; Lin et al., 2006) and subsequent transcription of enzymes involved in xenobiotic detoxification ultimately resulting cellular protection and maintenance of redox homeostasis (**Figure 1.9**).

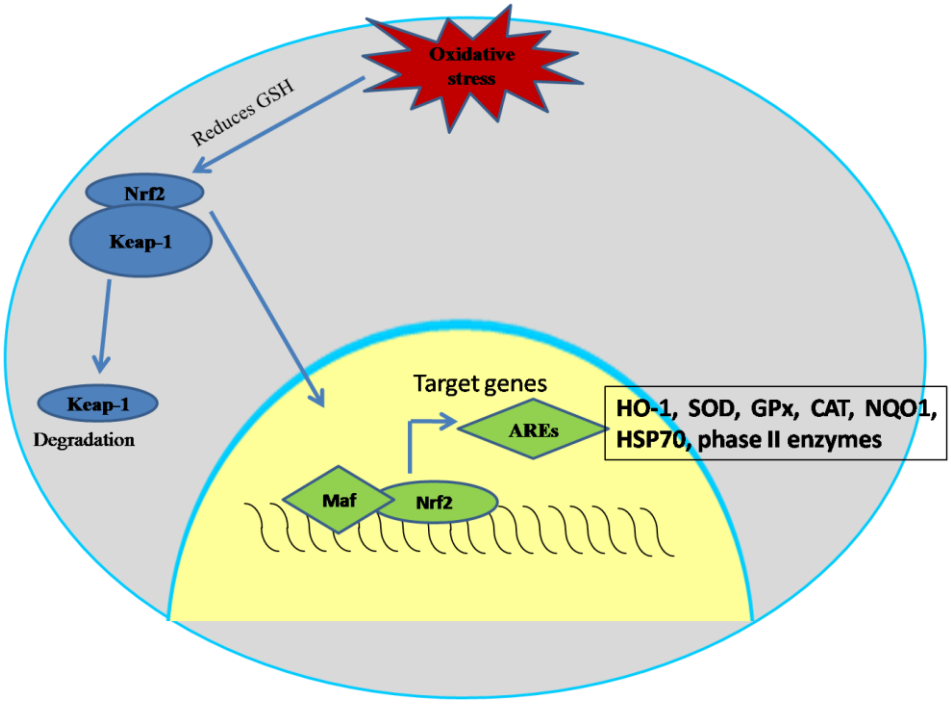


Figure 1.9. Schematic presentation of Nrf2 activation. In resting state, Nrf2 is sequestered in the cytosol via the Keap-1. Under oxidative stress intracellular GSH is depleted, resulting in conformational changes in Keap-1. This disables Keap1 from directing Nrf2 for degradation via the proteasome and enabling any newly synthesised Nrf2 to translocate into the nucleus. Nrf2 then associates with small

Maf protein forming a heterodimer which facilitates its capacity to bind to the ARE regions found in the regulatory domains of its Nrf2-regulated genes. Adopted from Copple et al 2008.

Prevention of cysteine modification within the BTB and IVR regions of the Keap-1 was found to facilitate Nrf2 ubiquitination (Zhang et al., 2004b), indicating that the targeting of these residues within the Neh2 domain is critical for Keap1-mediated repression of Nrf2. There is evidence that Keap1 acts as a "sensor" of chemical or oxidative stress, through its many cysteine residues. Furthermore, several recent reports have described the phosphorylation of Nrf2 as an event that is required for the nuclear export of the transcription factor (Jain and Jaiswal, 2006; Kaspar et al., 2009). Protein kinase C has been shown to phosphorylate Nrf2 in its Neh2 domain at Ser-40, disrupting the association between Nrf2 and Keap1 thus promoting the translocation of Nrf2 into the nucleus (Huang et al., 2002).

1.11.3 Nrf2 activators differentially modify Keap1

In response to electrophilic or oxidative insult, Nrf2 drives the induction of a battery of cytoprotective and anti-oxidant defence systems resulting in the maintenance of cellular redox homeostasis and prevention of cellular damage (Copple et al., 2010). It is possible that modification of any single cysteine residue in Keap1 may be sufficient to trigger the activation of Nrf2. Such a nonspecific triggering mechanism may underlie the chemical versatility of the Keap1-Nrf2 pathway, in terms of its capacity to "sense" and respond to a variety of structurally distinct molecules (Yamamoto et al., 2008). More specifically, it is possible that the modification of a single cysteine residue/group of residues within a critical domain of Keap1 provides the molecular trigger for Nrf2 activation (Zhang and Hannink, 2003). Administration of sulfhydryl reactive compounds, such as diethylmaleate, abolishes Keap1 repression of Nrf2 activity in cells and facilitates the nuclear accumulation of Nrf2 (Kwak et al., 2003). Additionally, the synthetic triterpenoids, CDDO-Methyl ester (CDDO-Me) and CDDO-Imidazolide (CDDO-Im), are found to be potent inducers of

heme oxygenase-1 and Nrf2/ARE signalling as well as up-regulating Nrf2 nuclear translocation (Yates et al., 2006). These triterpenoid derivatives provide an effective tool for investigating the role of Nrf2 in the maintenance of oxidative stress. Pre-treatment with CDDO-Me or CDDO-Im attenuates LPS-induced pro-inflammatory cytokine production and ROS generation in human PBMCs and neutrophils, respectively (Thimmulappa et al., 2007).

Because Nrf2–regulated cytoprotective genes protect from oxidative damage as well as suppress inflammation by modulating immune cell function, it is possible that the molecules which are able in activation of the Nrf2 pathway might prove to be suitable therapeutic strategy for intervention in sepsis as well as in systemic inflammatory response and other inflammatory disorders. (Thimmulappa et al., 2002).

1. 12 Role of Nrf2 in redox homeostasis and immune cell function

Nrf2 plays a vital role in maintaining cellular redox homeostasis through its ability to regulate the basal and inducible expression of a multitude of antioxidant proteins and detoxification enzymes (Nguyen et al., 2005). Nrf2 contributes to wide range of cellular functions including differentiation, proliferation, lipid synthesis and inflammation and there is an increasing evidence of association of aberrant expression and/or function of Nrf2 with different pathologies including cancer, neurodegeneration and cardiovascular disease (Osburn and Kensler, 2008). The development of Nrf2 knockout mice has provided principal understanding of the toxicological importance of this pathway, as these mice are found to be more vulnerable to the hepatic, renal, pulmonary, and neurotoxic consequences of acute or chronic exposures to environmental agents, drugs, inflammatory stresses, cigarette smoke and carcinogens (Kensler et al., 2007).

Nrf2 deficiency in mice have been shown to result in enhanced expression of several innate immune response proteins including specific cytokines, chemokines, and cell surface adhesion molecules and receptors. The lungs of Nrf2-deficient mice exhibited persistent cellular injury, pro-inflammatory IL-6 and TNF-α production, impaired endothelial and alveolar cell regeneration, and continual cellular infiltration by macrophages and lymphocytes which might impair lung innate immunity and promotes susceptibility to bacterial infection (Reddy et al., 2009). Disruption of Nrf2 causes regenerative immune-mediated hemolytic anemia (Lee et al., 2004). Furthermore, it was demonstrated that Nrf2-deficient mice exhibit prolonged inflammation during cutaneous wound healing (Braun et al., 2002). Through its influence on macrophages and neutrophil function, Nrf2 is implicated in the inflammatory response to bacterial infection, and protection against sepsis. Hence, Nrf2 has been described as a gene modifier for mounting appropriate innate immune responses (Thimmulappa et al., 2006).

Nrf2 has been revealed to influence DC redox homeostasis and on aspects of DC biology; however, the role of Nrf2 in DC immune function has not been fully elucidated. Evidence has shown that the intracellular redox status of the DC has a profound impact on its functions such as cell survival (Guyton et al., 1996; Nakamura et al., 1997b), DC activation and maturation (Kim et al., 2007; Matsue et al., 2003a; Verhasselt et al., 1999a) and DC cytokine production (Murata et al., 2002; Peterson et al., 1998; Sozzani et al., 2005). Additionally, ROS signalling and interactions with GSH have been implicated in the immunosenescence of DCs whereby a multitude of functions are impaired (Peters et al., 2009). Studies on DCs derived from Nrf2 deficient mice have identified the potential role of Nrf2 in the modulation of DC co-stimulatory receptor expression (Williams et al., 2008). Furthermore, Nrf2 deficient mice developed complex disease manifestations, exhibiting a lupus-like autoimmune syndrome with marked female dominance. This includes the presence of nuclear autoantigens, anti-double-stranded DNA

antibodies, multi-organ inflammation, immune complex deposition in blood vessels and glomerular nephritis (Ma et al., 2006).

However, the molecular and mechanistic basis for these altered co-stimulatory receptor expression of $Nrf2^{-/-}$ DCs are unknown and require investigation.

1.13 Heme oxygenases (HOs) in redox homeostasis

Heme is the product of catabolism of heme containing metalloproteins that has the potential to induce the generation of ROS (Figueiredo et al., 2007; Jeney et al., 2002). Heme chemically consists of an iron ion contained in the centre of a large heterocyclic organic ring called a porphyrin, made up of four pyrrolic groups joined together by methine bridges (protoporphyrin IX). Most of porphyrin-containing metalloproteins have heme as their prosthetic group which are known as hemoproteins, nevertheless not all porphyrins contain iron. Hemes are most commonly recognized as components of haemoglobin but they are also components of a number of other hemoproteins such as myoglobin, cytochrome, endothelial nitric oxide synthase and catalase (Maines, 1988). Excessive accumulation of heme within cells or in the extracellular environment can underlie pathological states such as neurodegenerative diseases (Alzheimer's disease, Parkinson's disease, and Huntington's disease), malaria (Prentice et al., 2007), and hemoglobinopathies (thalassemia and sickle Cell diseases) (Mariani et al., 2009).

Three HO isoforms: HO-1 (32 kDa), HO-2 (36 kDa), and HO-3 (33 kDa) have been described. HO-1 is normally expressed at low levels in most tissues but can be dramatically induced by a variety of stimuli, while HO-2 is constitutively expressed and is essentially un-inducible, and HO-3 is constitutive with limited tissue expression (Siow et al., 1999). The crystal structure of HO-1 consists of two helical folds with heme sandwiched between them (**Figure 1.10**). The proximal helix provides a heme iron ligand while the distal helix contains conserved glycines near the oxygen binding site that allows close contact between the helix backbone and

heme in addition to providing flexibility for substrate binding and product release (Schuller et al., 1999).

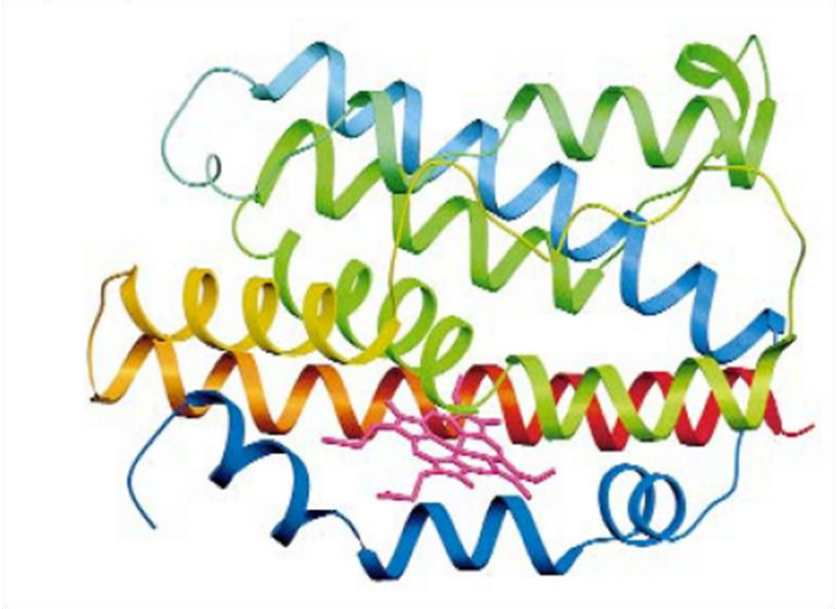


Figure 1.10. Schematic diagram of heme oxygenase-1. The N-terminus is blue and the C-terminus is red, with green in the middle. Adopted from Schuller et al. 1999.

Heme oxygenase-1 is a rate limiting microsomal membrane protein that catalyses the oxidative cleavage of heme molecules to yield equimolar quantities of biliverdin, carbon monoxide (CO), and iron. Biliverdin is subsequently reduced to bilirubin by the cytosolic enzyme biliverdin reductase (**Figure 1.11**), HO-1 is not a heme protein by nature, but it binds heme to form a 1:1 complex (Ryter et al., 2006).

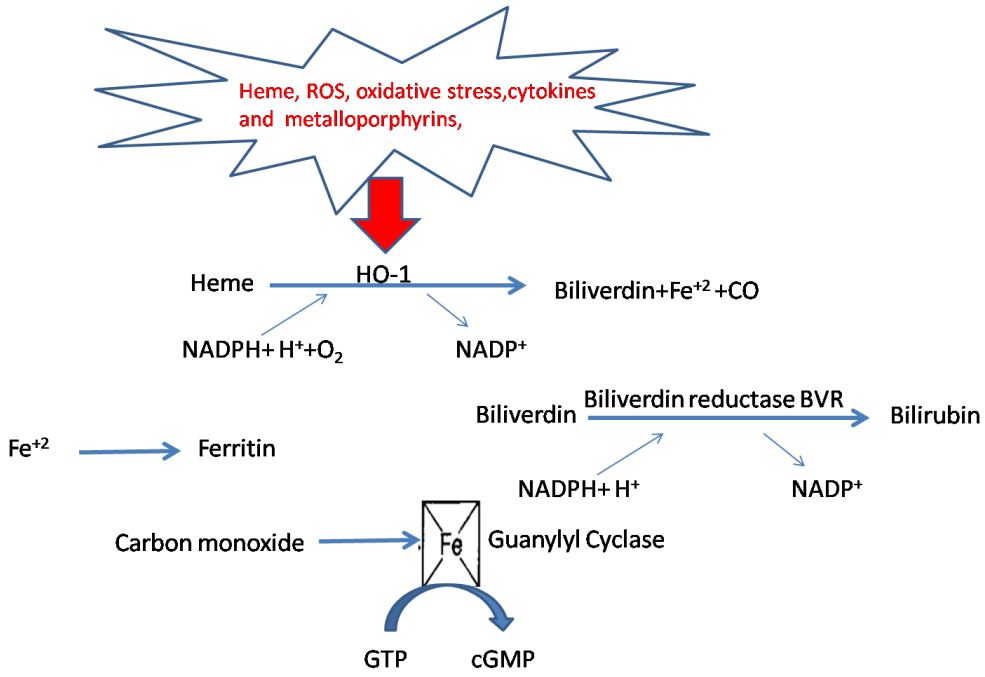


Figure 1.11. Schematic representation of heme degradation by heme oxygenase-1. Heme oxygenase-1 degrades heme to generate iron, carbon monoxide, and biliverdin-IX. Biliverdin-lX is subsequently reduced to bilirubin-IX by biliverdin reductase. Iron is sequestered by ferritin. Carbon monoxide can bind to the heme moiety of guanylyl cyclase to increase cellular levels of cGMP.

HO-1 is transcriptionally up-regulated as a sensitive cytoprotective protein by various types of oxidative stressors including its substrate heme and various environmental factors (Kitamuro et al., 2003). Nrf2 transcriptional activity was found to increase transcription of HO-1 expression (Sun et al., 2002). In the presence of low levels of heme, Bach1 (**Figure 1.12**) represses target genes with the Maf recognition elements (MAREs) in their regulatory regions by preventing activators such as Nrf2 from binding to MAREs.

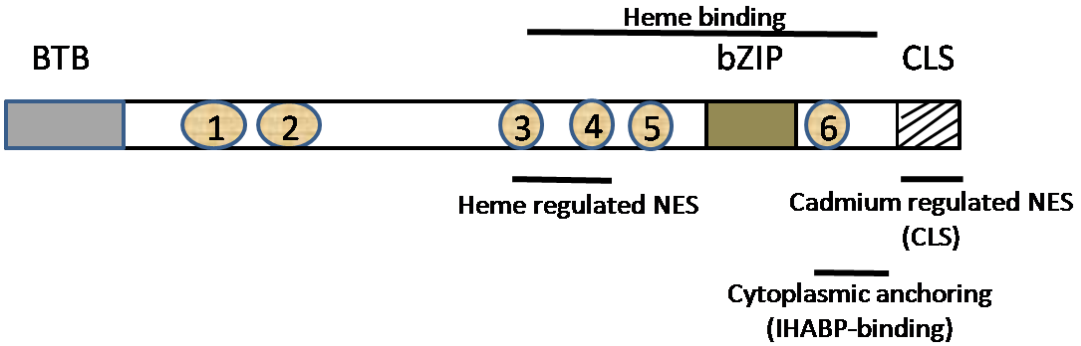


Figure 1.12. Schematic representation of Bach1 structure. Bach1 responds to heme and cadmium, two potent inducers of HO-1. Numbered circles represent cysteine-proline (CP) motifs. Subcellular localization of Bach1 is regulated by its interaction with the cytoplasmic, microtubule-associated, intracellular hyaluronic acid binding protein IHABP. Adopted from Igarashi et al 2006.

When heme levels build-up, this causes inhibition of the DNA-binding activity of Bach1, resulting in dissociation of Bach1 from the enhancers and de-repression of the target Nrf2 genes which means HO-1 induction by Nrf2 requires inactivation of the transcriptional repressor Bach1 (**Figure 1.13)** (Igarashi and Sun, 2006; Ogawa et al., 2001; Reichard et al., 2007). While Nrf2 is responsible for the production of HO-1 during oxidative insult, Bach1 plays the antagonistic role that is responsible for both positive and negative control over this critical gene (Sun et al., 2004). Indeed, HO-1 may be among the most critical cytoprotective mechanisms that are activated by heme as well as through non-heme cellular stress such as inflammation, ischemia, hypoxia, hyperoxia, hyperthermia, or radiation (Choi and Alam, 1996). Heme oxygenase-1 plays a critical role in counterbalancing oxidative stress and is an important modulator of physiological function with cytoprotective properties through inhibiting immune responses and inflammation *in vivo*. Indeed induction of HO-1 expression by pharmacologic activators or gene transfer has had therapeutic effects in a variety of conditions or disorders involving the immune system,

including transplantation and inflammatory disorders (Abraham, 2003; Soares et al., 2009).

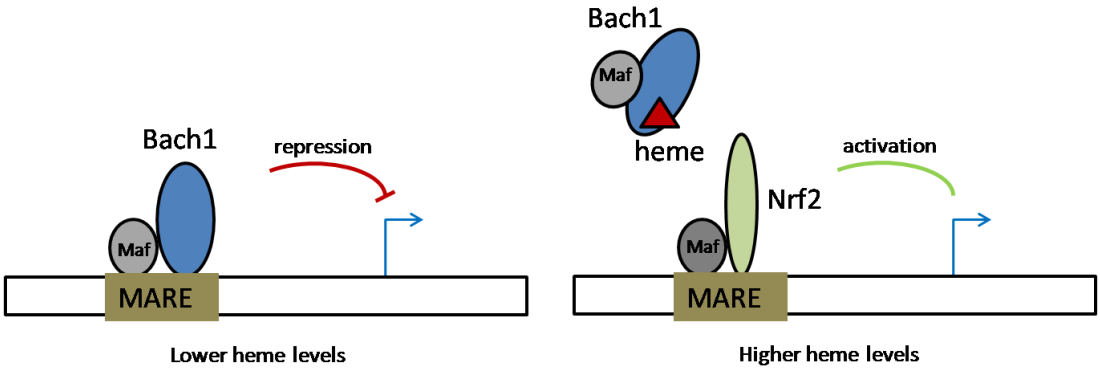


Figure 1.13. Transcriptional control of HO-1 expression. In the presence of lower concentrations of heme within a cell, Bach1 represses genes with MAREs together with small Maf proteins. In the presence of higher concentrations of heme, Bach1 is inactivated by binding to heme, leaving MAREs available for the activator class of factors such as Nrf2 or other MARE-binding proteins. In this way, heme directly regulates the balance of repression and activation of gene expression. Adopted from Ogawa et al, 2001.

Essential cytoprotective functions of HO-1 against oxidative stress could also be attributed to its enzymatic by-product CO and biliverdin (BV)/bilirubin (Chora et al., 2007; Ollinger et al., 2005). CO exerts anti-inflammatory, anti-apoptotic, and anti-proliferative effects (Kim et al., 2006). Additionally, exogenously administered CO has been shown to exert potent cytoprotective effects in various experimental models of oxidant stress including hemorrhagic shock (Zuckerbraun et al., 2005), hyperoxic lung injury (Otterbein et al., 1999), and endotoxemia (Kyokane et al., 2001). It has recently been suggested that bilirubin can be reconverted to BV when bilirubin is oxidized by peroxyl radicals and is then recycled by BV reductase to bilirubin (Baranano et al., 2002). This redox cycle might explain the antioxidant cytoprotective effects assigned to bilirubin and BV. The antioxidant activity of BV is

predominantly due to its ability to scavenge free radicals (Farrera et al., 1994; Stocker et al., 1987). Administration of BV provides effective protection in endotoxic shock (Sarady-Andrews et al., 2005) and decreases the production of inflammatory mediators such as cytokines and nitric oxide (NO) (Sawle et al., 2005).

Experimentally, metalloporphyrins have been found to either induce HO-1 gene expression, through up-regulation of transcription factor Nrf2 (for example, by cobalt protoporphyrin (CoPP)) (Shan et al., 2000), or inhibit HO-1 enzymatic activity (for example by zinc protoporphyrin (ZnPP) and tin protoporphyrin (SnPP)) (Chauveau et al., 2005; Kappas and Drummond, 1986; Maines, 1981).

In the context of DCs, HO-1 induction resulted in reduction of the LPS-induced expression of MHC class II and CD80 and CD86 along with decreased T cell proliferation and production of IFN-γ and IL-12 (Chauveau et al., 2005). Additionally, HO-1 expression was found to be down regulated upon DC maturation (Chauveau et al., 2005). However, the key signalling pathways through which HO-1 exerts its influence on DC function are unknown.

1.14 Aims of the study

The main focus of this study is to define the key signalling pathways involved in DC maturation and function that are subject to modulation by Nrf2 and HO-1.

Specifically, the thesis aims are to

1. Characterise the effect of Nrf2 activity on DC phenotype and function.
2. Define the effects of Nrf2 on NFκB, ERK, JNK and p38MAPK-CREB/ATF1 pathways in DCs
3. Test the requirement of ROS dysregulation in Nrf2-mediated effects on DC phenotype, function and signalling
4. Characterise the effect of HO-1 activity on DC phenotype, function and signalling

5. Examine the relevance of ROS disequilibrium in HO-1-mediated effects on DC phenotype and function

CHAPTER TWO

MATERIALS AND METHODS

2.1 Cell culture materials and reagents

Cell culture 10 cm microbiological petri dishes, Nunclon cell culture flasks, flat and round-bottom 96-well microtitre plates, 24 and 48 multi-well plates were purchased from Nunc [Thermo Fisher Scientific; MA, USA]. FACS tubes and sheath fluid were purchased from BD Bioscience [Oxford, UK]. Foetal bovine serum (FBS) and acrylamide gel cassettes were both purchased from Invitrogen [Paisley, UK]. Tris glycine, 4x Proto Gel Resolving Buffer, Proto Gel Stacking Buffer and 30% acrylamide: 0.8% (w/v) Bis-acrylamide stock solution were purchased from Gene Flow [Lichfield, UK]. Recombinant Granulocyte-macrophage colony-stimulating factor (GM-CSF) was purchased from Peprotech [London, UK]. Precision Plus protein Kaleidoscope standards, protein assay dye reagent and non-fat dry milk were all purchased from Bio-Rad [Hemel Hempstead, UK]. SB203580 (a specific inhibitor of p38MAPK which suppresses downstream activation of MAPKAP kinase-2) and PD98059 (a potent and selective inhibitor of MEK kinase) were both purchased from Cell Signalling Technology [Danvers, MA, USA]. BAY 11-7082 ((E)3-[(4-Methylphenyl) sulfonyl]-2-propenenitrile, a selective and irreversibly inhibitor of NF-κB activation) and cAMPS-Rp ((*R*)-Adenosine, cyclic 3',5'-(hydrogenphosphorothioate) triethylammonium, a competitive antagonist of cAMP-induced activation of PKA) was obtained from Merck Chemicals Ltd [Nottingham, UK] and Tocris Bioscience [Bristol, UK], respectively. Tin protoporphyrin IX dichloride (SnPP IX) and cobalt protoporphyrin IX chloride (CoPP) were both purchased from Frontier Scientific [Newark, USA]. All chemicals and reagents were of analytical grade and purchased from Sigma-Aldrich [Dorset, U.K.], unless otherwise stated.

2.2 Antibodies

Fluorochrome conjugated antibodies: the intracellular fluorescent dye, carboxyfluorescein succinimidyl ester (CFSE) and fluorescein (FITC)-dextran [40,000 MW], PE-Cy®5.5 conjugated anti-mouse CD11c (N418), were purchased from Invitrogen [Paisley, UK]. FITC-conjugated anti-mouse CD40 (Clone 3/23), FITC-conjugated anti-mouse CD86 (GL1), PE-conjugated anti-mouse I-A/I-E (M5/114.15.2), FITC-conjugated Annexin V and PE-Cy®5.5 conjugated Propidium iodide (PI) detection kit were purchased from BD Bioscience [Oxford, UK]. Dihydroethidium (DHE), the fluorescent ROS indicator, was purchased from Sigma-Aldrich [Dorset, UK].

Primary antibodies for Western immunoblotting (IκBα; p-p65; p-ERK1/2; ERK1/2; p-p38; p38; p-JNK; JNK; p-CREB/ATF1; CREB and Bcl-2) were purchased from Cell Signaling Technology [MA, USA]. p65 and tubulin were purchased from Santa Cruz Biotechnologies [CA, USA]. Actin and HO-1 were both purchased from Abcam [Cambridge, UK]. The secondary horseradish peroxidase (HRP) conjugated anti-mouse IgG and anti-rabbit IgG antibodies were purchased from Cell Signaling Technology [MA, USA].

2.3 Cell lines

The human leukaemic Jurkat T cell line, E6.1 was purchased from American Type Culture Collection (ATCC) [Middlesex, UK]. These cells were utilised for the dendritic cell phagocytosis assay. Cells were cultured in CM at a density of 0.5 $x10^6$ cells / ml in nunclon cell culture flasks and media was changed every two days until required for functional assays.

2.4 Mice

Black C57BL6/SV129 wild type and Nrf2 knockout mice ($Nrf2^{-/-}$) were purchased from Riken BRC [Ibaraki, Japan] (Itoh et al., 1997a). Nrf2 colonies were maintained as heterozygous breeders and homozygous offspring were genotyped before use. C57BL/6 mice homozygous for the H-$2D^b$-restricted TCR-$\alpha\beta$ transgene, F5, which recognises NP-68, a nonameric peptide derived from influenza virus A/NT/60/68 nucleoprotein were a kind gift from Dr James Matthews [Cardiff; Wales] and housed under controlled conditions at the Biomedical Services Unit, University of Liverpool. Mice were given free access to food and water and housed at a temperature of between 19°C - 23°C under 12 h light/dark cycles. All animals were bred under pathogen free conditions. Protocols described herein were undertaken in accordance with criteria outlined in licenses granted under the Animals (Scientific Procedures) Act 1986 (PPL 40/2937 and PPL 40/2544) and approved by the Animal Ethics Committee of the University of Liverpool. The NP68 peptide (^{366}ASNENMDAM^{374}) used to stimulate F5 CD8 T cells was synthesised by Peptide Protein Research Ltd [Hampshire, UK].

2.5 Generation of mouse bone marrow-derived dendritic cells (DCs)

Bone marrow cells were flushed from femora and tibiae of wild type and $Nrf2^{-/-}$ mice. Cells were washed and cultured in complete medium (Roswell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with 10% FBS, 50 μM 2-ME, 2 mM HEPES buffer, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU/ ml streptomycin and 100 μg/ ml penicillin) in the presence of 20 ng/ml GM-CSF at a density of 3 x 10^6 cells/ plate in 10cm microbiological petri dishes. Cultures were kept in a fully humidified incubator at 37°C under 5% CO_2, media was then replenished on day 3 with the addition of 20 ng/ ml GM-CSF and differentiated DCs

were used between days 6 or 7. An exception is for DC proliferation assay, as explained in 2.13, where DCs on day 3 were harvested from cell culture petri dishes, counted and re-seeded in 96-well round-bottom microtitre plates at different cell densities with20 ng/ml GM-CSF.

2.6 Viable cell count by Trypan Blue exclusion

Cell suspensions were mixed with an equal volume of 0.2% w/v Trypan Blue in HBSS. The samples were then pipetted into the counting chambers of a haemocytometer [Weber Scientific International Ltd.; Lancing, UK] and counted at 100X magnification. Dead cells absorb the Trypan Blue dye and appear blue, whereas viable cells exclude the dye and appear clear.

2.7 Immunomagnetic separation of $CD11c^{+ve}$ DCs (For ELISA only)

Purified CD11c positive wild type and $Nrf2^{-/-}$ bone marrow derived DCs were isolated using MACS LS^{+} immunomagnetic separation columns [Miltenyi Biotec, Germany]. Cells were washed and resuspended at a density of in 1 x 10^{8} cells per 400 µl of ice cold MACS Buffer (HBSS without phenol red supplemented with 2 mM EDTA and 0.5% bovine serum albumin (BSA)). Cells were magnetically labelled using anti-CD11c conjugated magnetic microbeads [Miltenyi Biotec, Germany] 20 µl per 1 x 10^{6} cells, and refrigerated for 15 minutes. Cells were washed with MACS buffer (2 ml per 1 x 10^{6} cells), centrifuged at 300 g for 10 minutes and resuspended in 500 µl MACS buffer. MACS LS^{+} Separation Columns were attached to MidiMACS magnets and set up on a MidiMACS stand. The columns were washed three times with 3 ml of MACS buffer. The magnetically labelled cell suspensions were pipetted onto the column. Unlabelled cells were collected as the negative fraction whereas labelled cells were retained within the column. A thorough elution of the negative fraction

was facilitated by washing the columns three times with 3 ml of MACS buffer. The columns were then removed from the magnet and flushed with 5 ml of MACS buffer using a column plunger. This procedure yielded positive cell populations of approximately 90 % purity (data not shown).

2.8 Co-stimulatory receptor expression by flow cytometry

For DCs phenotyping, cells were scraped from petri dishes and washed in HBSS, counted and dispensed into FACS tubes at a density of 1 x 10^6 cells/ tube. Cells were washed with stain buffer (sheath fluid supplemented with 2% FBS and 2 mM ethylene diamine tetra acetic acid (EDTA)). Washed cells were resuspended in 50 μl stain buffer and incubated in the dark at 4°C for 10 minutes. Appropriate amounts of antibodies were dispensed to each tube in 50 μl stain buffer and tubes were incubated for 30 minutes in the dark at 4°C. After incubation, cells were washed in 4 ml sheath fluid and resuspended in 700 μl sheath fluid prior to analysing on a BD FACSCanto II [BD Biosciences; Oxford, UK].

Post acquisition analysis of flow cytometry data (FCS) files were conducted using Cyflogic v.1.2.1 software [CyFlo Ltd, Finland].

2.9 Isolation of F5 CD8 T cells

Spleens were harvested from from F5 TCR transgenic mice and homogenised using Jencons Uniform Grade B-24 Tissue Homogeniser [Jencons Scientific Ltd., Leighton Buzzard, UK]. Dispersed tissue was then filtered through 40 μm gauge Nylon Cell Strainers [Falcon, UK]. Cells were washed in HBSS and spun at 300 g for 5 minutes. After centrifugation, pelleted cells were treated with red blood cell (RBC) lysis buffer (8.3 g/L ammonium chloride in 0.01 M Tris-HCl buffer) for 6 minutes at RT

(25 °C) to remove red blood cells, followed by a final wash/centrifugation cycle. Lymphocytes were then resuspended in complete medium.

2.10 F5 CD8 T cell proliferation assay

F5 CD8 T cell proliferation was quantified as described in (Johnson et al., 1999). Isolated splenocytes from F5 TCR mice were processed and plated out in quadruplicate at a density of 1 x 10^5 cells/ well in 96-well round-bottom microtitre plates in complete medium. Dendritic cells were pre-pulsed with a concentration range of NP68 for 2 hours at 37°C, washed and co-cultured with F5 T cells at a density of 5000 cells/ well. Cultures were incubated at 37°C under 5% CO_2 for 72 hours and ^{3}H-Thymidine added for the last 16 hours. Cultures were harvested onto glass fibre filter mats using a Tomtec Mach III harvester [Tomtec; Connecticut, USA]., Dried filter mats sealed with solid scintillant [Perkin Elmer; Cambridge, UK] were read on a scintillation counter [MicroBeta Trilux; Perkin Elmer; Cambridge, UK].

2.11 Measurement of reactive oxygen species (ROS) levels

Intracellular ROS levels were determined by measuring the levels of fluorescence generated by the rapid oxidation of DHE to its fluorescent oxidised form, ethidium bromide in the presence of ROS (emission: 420nm). Intracellular superoxide levels of DCs were determined by labelling DCs with DHE according to (Bindokas et al., 1996; Burnaugh et al., 2007) with minor modifications. Briefly, 5 x 10^5 DCs were washed and resuspended in serum-free RPMI 1640. Cells were treated with or without LPS or H_2O_2 for 10 minutes. Cells were then washed and resuspended in HBSS without red phenol before 10 μM of DHE was added and incubated at 37°C for 10 minutes. Cells were analysed by flow cytometry (see section 2.8).

2.12 Apoptosis assay

Treated and untreated DCs (1 x 10^5 cells per FACS tube) were washed with 1X binding buffer (contained in the Annexin V kit), and incubated with fluorescently labelled FITC-conjugated Annexin V or Propidium iodide (PI) plus 1X binding buffer in the dark at RT for 15 minutes. At the end of the incubation, 400 μl of 1X Binding Buffer was added to each tube and analysed by flow cytometry within 1 h.

2.13 DC proliferation assay

On day 3, DCs were harvested from cell culture petri dishes, counted and re-plated in 96-well round-bottom microtitre plates at different cell density in complete medium supplemented with 20 ng/ ml GM-CSF and ^{3}H-Thymidine in a total volume of 180 μl per well. At day 5, 6 and 7, cultures were harvested onto glass fibre filter mats using a Tomtec Mach III harvester and read on a scintillation counter.

2.14 ATP activity assay

This assay was used to determine the number of viable cells in culture based on the quantitation of the ATP present, which reflects the amount of metabolically active cells. On day 7, DCs were harvested from cell culture petri dishes, counted and re-plated in 96-well flat-bottom microtitre plates at a density of 25 x 10^3 cells per 100 μl per well in complete medium. According to CellTiter-Glo® Assay instructions, the CellTiter-Glo® Buffer was mixed with the CellTiter-Glo®Substrate to reconstitute the lyophilised enzyme/substrate mixture. CellTiter-Glo® Reagent (100 μl) was mixed into the plate wells and left for 10 minutes prior to quantifying the luminescence signal on a Varioskan plate reader [Thermo Scientific; MA, USA].

2.15 Detergent lysis of DCs

On day 6, DCs were harvested and resuspended in serum free RPMI 1640 at a density of 10-20 x 10^6 cells/ condition. They were either stimulated with 1 μg/ ml LPS for indicated time points at 37°C; or left unstimulated on ice with or without kinase inhibitors. Cold complete media was added to quench the reaction and the cells were then centrifuged and lysed in 1 ml cold 0.5% Nonidet P-40 lysis buffer (0.5% Nonidet-P40, 1 mM Na_3VO_4, 25 mM NaF, 150 mM NaCl, 50 mM Tris-HCl pH 8.0, 1 mM EDTA) supplemented with protease inhibitors (1 mM PMSF, 10 μg/ ml Aprotinin, 10 μg/ ml Leupeptin and 10 μg/ ml Pepstatin A) which were added to the lysis buffer immediately prior to cell lysis. Cells were left in the lysis buffer on ice for 30 minutes and then centrifuged at 16000 g for 15 minutes at 4°C. Supernatants were removed and transferred into new pre-cooled eppendorfs, aliquoted into small volumes and kept in -80°C until further use.

2.16 Nuclear extraction of DCs

Nuclear Lysates were generated from cell samples using NE-PER nuclear and cytoplasmic extraction kit [Fischer Scientific, Loughborough, UK] via strict adherence to manufacturer's instructions. Briefly, 10 x 10^6 DCs/condition were centrifuged, supernatant was removed and pellets resuspended in 100 μl cytoplasmic extraction reagent I (CER I) plus protease inhibitors. Cells were left on ice for 10 minutes after which 5.5 μl CER II was added to each eppendorf, vortexed and left on ice for a further 1 minute. Samples were then spun at 16,000 g at 4°C for 5 minutes and the supernatant containing cytoplasmic fractions were removed and stored at -80°C for future use. The remaining pellets were resuspended in 50 μl nuclear extraction reagent (NER) and left on ice for 45 minutes with intermittent vortexing every 15 minutes. Cells were then spun at 16,000 g at 4°C for 10 minutes

and the supernatant containing nuclear fractions was carefully removed and stored at -80°C for future use.

2.17 Protein content determination via Bradford assay

Whole cell lysates and/or nuclear extracts from cell samples were generated as appropriate to each experimental requirement. The total protein content of samples was determined using Protein Assay Dye Reagent,] according to the manufacturer's instructions. Based on the method of Bradford (Bradford, 1976), this assay relies on the binding of Coomassie Brilliant Blue G-250 dye to basic and aromatic amino acids, resulting in a change in colour of the dye (red to blue). This change in the dye colour is proportional to the amount of bound dye and can be measured in the absorbance at 570 nm with a MRX plate reader [Dynex; Sussex, UK]. Total protein content of samples can be calculated from the absorbance values of a 0.25-5 μg BSA standard curve prepared for each assay.

2.18 Western blotting analysis

Whole cell lysates (5-10 μg of protein determined by Bradford assay) were denatured via the addition of 5 μL loading buffer (70% (v/v) NuPAGE sample loading buffer, 30% (v/v) NuPAGE reducing agent) and incubated at 90°C for 5 minutes. Samples were loaded onto NuPAGE Novex bis-tris polyacrylamide gels, alongside PrecisionPlus protein Kaleidoscope standards. Samples were resolved by electrophoresis in a XCell Surelock mini-cell from Invitrogen [Paisley, UK], using a 3-(N-morpholino) propanesulphonic acid (MOPS) running buffer (50 mM MOPS, 50 mM Tris base, 3.5 mM sodium dodecyl sulphate, 1mM EDTA, 0.25% (v/v) NuPAGE antioxidant), at 90 Volts for 10 minutes, followed by 60 minutes at 170 Volts. Resolved proteins were transferred to polyvinylidene difluoride (PVDF) membranes

[Bio-rad; Hemel Hempstead, UK] at 200 mA for 90 minutes with ice. To check whethere the transfer process was successful, membranes were stained for 30 seconds with Ponceau S solution. Membranes were blocked for 1 h at RT in tris-buffered saline (TBS; 150 mM NaCl, 50 mM Tris base, pH 7.6) containing 0.1% (v/v) Tween-20 and 10% (weight/volume; w/v) non-fat dry milk. Blocked membranes were washed in TBS-0.1% Tween-20 and probed overnight at 4°C with appropriate primary antibodies (1:1000 to 1:20000 in TBS-0.1% Tween-20 containing 2% (w/v) BSA). Following several washes in TBS-0.1%Tween-20 for 1 h, membranes were probed for 1 h with goat anti-rabbit (1:5000 to 1:20000 in TBS-0.1% Tween containing 2% (w/v) BSA) horseradish peroxidise (HRP)-conjugated secondary goat anti-rabbit or anti-mouse IgG peroxidase conjugate. Immunoblots were visualised with Western Lightning chemiluminescence reagents [Perkin Elmer, Cambridge, UK] and exposed to Hyperfilm ECL [GE healthcare Life Sciences, Chalfont St Giles, UK] under darkroom conditions, using a Kodak BioMax MS intensifying screen [Healthcare Amersham; Thermo Fisher Scientific; MA, USA]. Blots were developed using Kodak developer and fixer solutions (Kodak; Hemel Hempstead, UK).

In order to ensure equal loading across gels, membranes were probed with mouse anti-β-actin primary antibody (1:10000 in TBS-0.1% Tween containing 2% (w/v) BSA) or mouse anti-tubulin primary antibody (1:5000 in TBS-0.1% Tween containing 2% (w/v) BSA) and goat IgG anti-mouse or anti-rabbit HRP-conjugated secondary antisera.

Films were scanned using a GS-710 calibrated imaging densitometer [Bio-rad; Hemel Hempstead, UK] and immunoreactive band volumes were quantified using TotalLab 100 software, in accordance with the manufacturer's instructions, and normalised to total protein.

Target protein	Primary antibody concentrations	Secondary antibody type and concentrations
Bcl-2	1:1000, 4°C o/n	Rabbit 1:5000, 37°C 45 mins
IκB α	1:1000, 4°C o/n	Rabbit 1:5000, 37°C 45 mins
Actin	1:20000, 37°C 30 mins	Rabbit 1:20000, 37°C 30 mins
p-p65	1:1000, 4°C o/n	Rabbit 1:5000, 37°C 45 mins
p65	1:1000, 4°C o/n	Mouse 1:5000, 37°C 45 mins
p-ERK1/2	1:1000, 4°C o/n	Rabbit 1:5000, 37°C 45 mins
ERK1/2	1:1000, 4°C o/n	Rabbit 1:5000, 37°C 45 mins
p-JNK	1:1000, 4°C o/n	Rabbit 1:5000, 37°C 45 mins
JNK	1:1000, 4°C o/n	Rabbit 1:5000, 37°C 45 mins
p-p38	1:1000, 4°C o/n	Rabbit 1:5000, 37°C 45 mins
p38	1:1000, 4°C o/n	Rabbit 1:5000, 37°C 45 mins
Tubulin	1:1000, 4°C o/n	Mouse 1:5000, 37°C 45 mins
p-CREB/p-ATF1	1:1000, 4°C o/n	Rabbit 1:5000, 37°C 45 mins
CREB	1:1000, 4°C o/n	Rabbit 1:5000, 37°C 45 mins
HO-1	1:1000, 4°C o/n	Rabbit 1:5000, 37°C 45 mins

Table 2.1 Summary of Western blotting antibodies used in the study. o/n= over night.

2.19 Reprobing of blotted membranes

Reprobing of membranes for other proteins following initial immunoblotting and ECL developing was done by firstly stripping the membrane of antibodies. For this purpose, membranes were incubated in Stripping Buffer (100 mM 2-ME, 2% SDS, 62.5 mM Tris-HCl pH6.8) for 30 minutes at 55°C. The membranes were transferred into TBS-0.1% Tween-20, washed three times for 10 minutes each and blocked in blocking buffer (10% non-fat dry milk in TBS-0.1% Tween-20) for 30 minutes. The membranes were subsequently immunoblotted for specific proteins and developed as described earlier.

2.20 Measurement of NF-κB activity

NF-κB activity was determined using the EZ-Detect™ NF-κB p65 Transcription Factor Kit [Pierce Biotechnology; Thermo Fisher Scientific; MA, USA] following the manufacturer's instructions. This is an ELISA based technique using nuclear lysates and it is provided with a 96-well in multi-strips format with the bound biotinylated-consensus sequence for the p65 subunit. The biotinylated-consensus duplexes will bind only to the active form of the transcription factor subunit p65. This system is designed to improve result specificity. Nuclear extracts (20 μg) were added to the pre-coated wells and incubated at RT for 1 h. The plate was then washed using wash buffer and the anti-p65 primary antibody was added to the wells and incubated for 1 h on a shaker at RT. This is followed by another round of washing and incubation with HRP conjugated secondary antibody for another 1 h at room temperature without shaking. After washing, a luminol based chemiluminescent substrate provided with the kit was added to the wells and the signals were detected using Varioskan plate reader. Results were expressed as relative luminescence units (RLU).

2.21 Histone deacetylase (HDAC) activity assay

HDAC activity of DC nuclear extracts was determined using the HDAC Assay Kit [Millipore; Massachusetts, USA] following the manufacturer's instructions. HDAC assay buffer x 2 (10μl) was dispensed into each well of the 96-well round-bottom microtitre plate followed by the addition of 10 μl DC nuclear extracts samples into the wells which were allowed to equilibrate at 37°C. Subsequently, 10 μl of 4 mM HDAC Assay Substrate was added to the wells and mixed thoroughly and incubated at 37°C for 60 minutes. At the end of the incubation, 20 μl of the diluted activator solution was added to each well, mixed thoroughly and incubated for 10 minutes at room temperature, and then absorbance read at 405 nm (OD 405) using Varioskan.

2.22 Enzyme-Linked Immunosorbent Assay (ELISA)

On day 6, DCs were harvested, washed with HBSS and $CD11c^{+ve}$ DCs were magnetically isolated using CD11c beads. These DCs were then seeded onto 24-well plates in duplicate at a density of 8 x 10^5 DCs per ml with a total volume of 2 ml/ well. After 48 h, cell-free cultures supernatants from untreated and treated groups were collected for measuring cytokines concentrations by Quantikine ELISA (R&D system; Abingdon, UK) according to manufacturer's protocol.

Briefly, 100 µl of standard and samples solutions was added to a 96-well polystyrene microplate pre-coated with polyclonal antibody specific for mouse cytokine. Solutions were mixed by gently tapping the plate frame for 1 minute and then cover with the adhesive strip provided and incubated for 2 h at room temperature with mild agitation. After that, each well was aspirated and washed with wash buffer (0.5% Tween-20 in 1x PBS), the process was repeated four times for a total of five washes. After the last wash, 100 µL of mouse cytokine conjugate was added to each well and incubated for 2 h at RT. When the incubation time finished, the wash steps were repeated as above and then 100 µL of substrate solution was added to each well and the wells incubated for 30 minutes at RT with protection from light. At the end of the 30 minutes, 100 µL of stop solution was added to each well and the plate gently tapped to ensure thorough mixing and then the optical density of each well was determined within 30 minutes using a microplate reader Varioskan set to 450 nm. Based on the raw OD data obtained, a standard curve was plotted and the concentrations of each cytokine were then calculated.

2.23 DC endocytosis assay

Endocytic ability of iDCs was determined using Dextran^{FITC} as previously described (Sallusto et al., 1995b). Briefly, iDCs were harvested and resuspended at a density of 1 x 10^6 cells in 100 μl complete medium. DCs were incubated with 0.5 μg/ μl Dextran^{FITC} [40,000 MW] for indicated time points at 37°C. Cells were washed twice in stain buffer (2% FBS, 2 mM EDTA in PBS), stained with anti-CD11c^{TC} for surface expression of CD11c as described above and analysed by flow cytometry.

2.24 DC phagocytosis assays

Apoptotic thymocytes were generated by harvesting thymi from C57BL/6 mice followed by tissue homogenisiation, washing and cell resuspension in complete media (3 x 10^6 cells/ ml). Cells were incubated with 1 μM dexamethasone at 37°C under 5% CO_2 for 18 h. The proportion of apoptotic cells were determined by staining with Annexin-V yielding over 90% of apoptotic cells (data not shown). Apoptotic thymocytes were labelled with CFSE (4 μM) for 20 minutes at 37°C prior to washing and co-culturing with 2 x 10^6 cells/ well (24-well plate) plate adherent DCs at a ratio of 2:1 for 2 h at 37°C under 5% CO_2 and 4°C as a control. Cells were harvested and washed in stain buffer and stained with anti-CD11c^{TC} prior to analysis by flow cytometry.

To assess the phagocytosis of necrotic cells, Jurkat T cells were fluorescently labelled with 0.5 μM CFSE for 30 minutes and necrosis was induced via snap freezing in liquid nitrogen. More than 90% of cells were necrotic as determined by trypan blue exclusion (data not shown). Necrotic cells were co-cultured with DCs as above. Cells were harvested and washed in stain buffer and stained with anti-CD11c^{TC} prior to analysis by flow cytometry.

2.25 Statistical analysis

Where appropriate, experiments were performed at least in triplicate, and all experiments were replicated on separate occasions. Depending on the results set, data are expressed as either mean ± standard deviation of the mean (SD) or mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) with Tukey's post-test, Mann-Whitney *U* test, or Student's t-test were used to assess the significance of any differences in the data compared to appropriate controls. A two-sided *P* value of ≤0.05 was considered to be statistically significant.

CHAPTER THREE

REGULATION OF DC PHENOTYPE, FUNCTION, AND SIGNALLING PATHWAYS BY NRF2

3.1 INTRODUCTION

Dendritic cells (DCs) are potent antigen presenting cells (APCs) that serve as sentinels which initiate and modulate immune responses (Cella et al., 1997b; Hart, 1997). They possess molecular sensors and antigen-processing machinery required to recognize pathogens and integrate chemical information ultimately leading to an orchestrated immune response that is of appropriate magnitude, quality, and specificity (Drozina et al., 2005; Jones, 1997; Steinman et al., 1999). The activation of naive T cells by DCs requires both signal 1 (TCR signals via MHC class I and II) and signal 2 (co-stimulatory signals via CD80, CD86 and CD40) as well as signal 3 from cytokines which help to elicit distinct T cell responses (Bachmann et al., 1999b; Banchereau et al., 2000). Up-regulation in the expression of co-stimulatory cell surface molecules and increased cytokine secretion are associated with transformation of DCs from an immature to a mature state (Banchereau and Steinman, 1998; Caux et al., 1994; Inaba et al., 1994). Many signalling pathways are involved in DC maturation and these are primarily initiated through the binding of Toll-like receptors (TLR) to their ligands, usually bacterial or viral-derived proteins and nucleic acids. Engagement of TLRs triggers a complex network of signalling molecules and adaptor proteins that will ultimately modulate DC responses. Each individual TLR can activate common and unique transcription factors through different intracellular signalling pathways (Kawai and Akira, 2005). Of the 13 known mammalian TLRs and their ligands, lipopolysaccharide (LPS), which activates TLR4, is the most commonly used ligand for the maturation of DCs *in vitro*. All these signalling networks cooperate, integrate, and finally converge onto major pathways such as that of the nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs) (Agrawal et al., 2003; Zhong et al., 2006).

The production of reactive oxygen species (ROS) that possess high chemical reactivity is due to the incomplete reduction of oxygen during aerobic metabolism

and from exposure to some natural and synthetic toxicants (D'Autreaux and Toledano, 2007; Davies, 2000; Yu, 1994). ROS include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^\bullet$). ROS can also function as intracellular signalling molecules or serve as diffusible signals that modulate various intracellular signalling pathways by chemically reacting with specific atoms of target proteins leading to covalent protein modifications (Forman and Torres, 2002; Nathan, 2003).

Intracellular redox homeostasis is maintained through a number of fast, precise, and highly dynamic cytoprotective defence measures that counterbalance the increment in ROS levels. The functionality of these adaptive cytoprotective cellular defence systems are primarily governed by the activity of the redox responsive transcription factor Nrf2 (D'Autreaux and Toledano, 2007). Under conditions of chemical/oxidative stress, Nrf2 is able to evade Keap1-mediated repression, accumulate within the nucleus and mediate transactivation of ARE-dependent genes (Copple et al., 2008). Therefore, Nrf2 may serve as a master regulator of the ARE-driven cellular defence system against oxidative stress. In addition, Nrf2 can regulate the balance between cell quiescence and proliferation and self-renewal and differentiation (Lee et al., 2005).

In the context of DC biology, Nrf2 has been found to regulate co-stimulatory molecule expression and modulate cytokine secretion (Rangasamy et al., 2010). In addition to that, Nrf2-mediated signalling mechanisms alter the DCs response to common environmental allergens, which may contribute to susceptibility to allergic diseases (Williams et al., 2008).

However, intracellular signalling mechanisms that are involved in Nrf2-mediated regulation of DC phenotype and function have not been defined. In addition, the relevance of altered ROS levels arising as a result of Nrf2 deficiency, in the context of DC activation and function has not been investigated.

The hypothesis of the work described in this chapter is that Nrf2 affects DC biology through its effects on the key intracellular signalling pathways, NF-κB and MAPK pathways. Through utilisation of the Nrf2 experimental mouse model, we also aimed to define the requirement of ROS dysregulation in Nrf2-mediated effects on DC phenotype, function, and signalling.

3.2 RESULTS

3.2.1 Loss of Nrf2 has variable effects on DC proliferation depending on culture conditions

Dendritic cells (DCs) develop from bone marrow precursors and are distributed in limited numbers throughout peripheral tissues and lymphoid organs (Steinman, 1991). Despite the fact that DCs represent only a small percentage of mononuclear leukocytes, they play a major role in initiating and regulating immune responses (Banchereau et al., 2000). Generation and distribution of sufficient numbers of DCs is required for optimal immune functions.

Previous studies have shown that Nrf2 controls mechanisms which regulates stem cell proliferation in high-turnover tissues such as bone marrow and intestine, and is apparent by the excessive expansion of haematopoietic progenitors from $Nrf2^{-/-}$ bone marrow precursors (Hochmuth et al., 2011; Tsai et al., 2013). However the impact of loss of Nrf2 on DC proliferation and differentiation from bone marrow progenitors has not been evaluated. In the current study, we tested the hypothesis that loss of Nrf2 results in increased DC proliferation. In order to examine this, murine $Nrf2^{-/-}$ and $Nrf2^{+/+}$ bone marrow progenitor cells were initially seeded in culture medium at increasing cell densities and cultured in GM-CSF over various time points. The final differentiated DC numbers for both genotypes were then

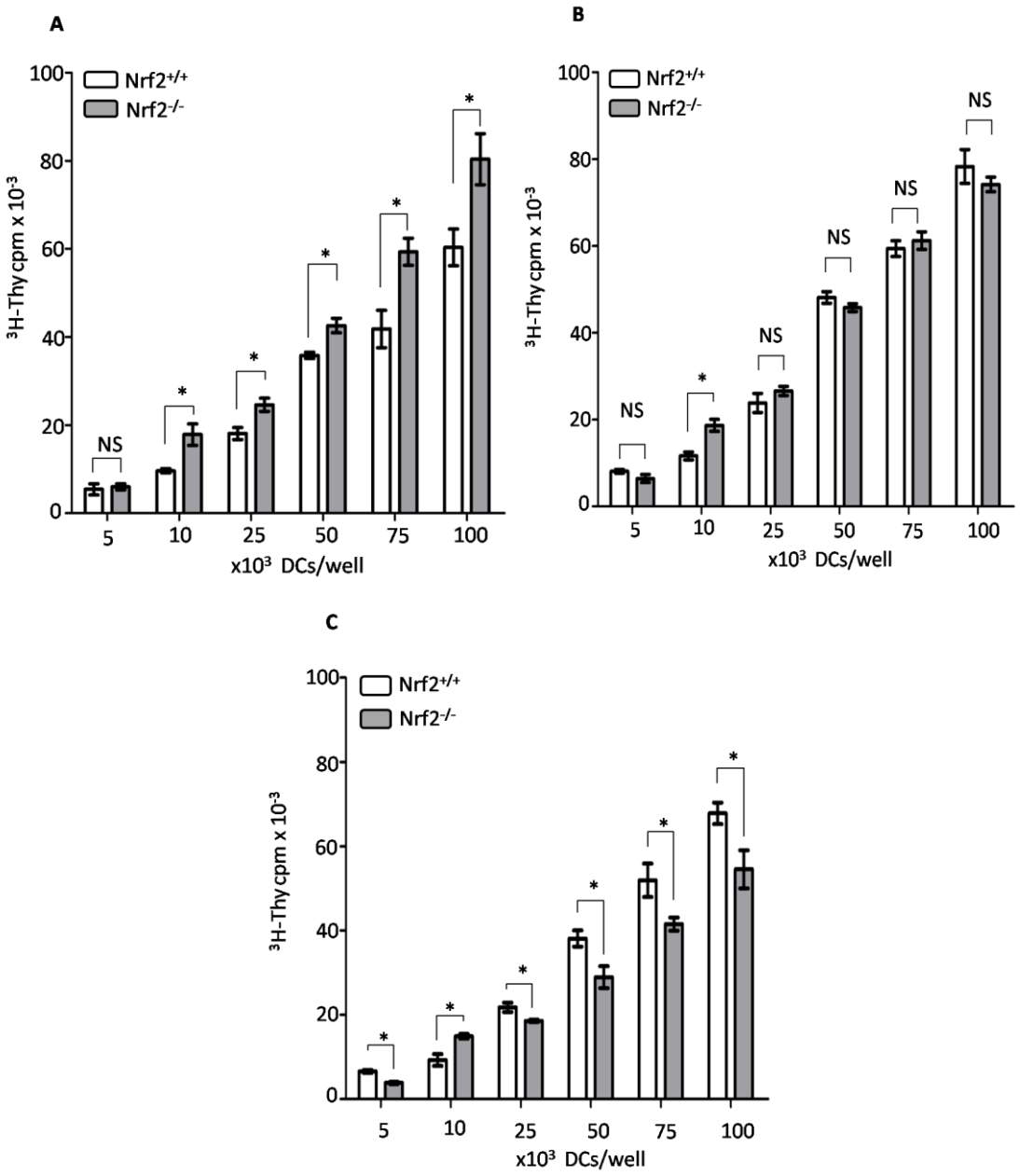


Figure 3.1. Nrf2 modulation of DC proliferation depends on culture conditions. $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs were plated at indicated cell densities. [^{3}H]Thymidine (^{3}H-Thy) was added at day 4. Proliferation of iDCs was determined by scintillation counting of incorporated [^{3}H]Thymidine at day 5 (**A**), day 6 (**B**) and day 7 (**C**). Data are presented as average [^{3}H]Thymidine scintillation counts ± S.D. Statistical significance was tested by one-way analysis of variance (*, $p<0.05$). Data are derived from three independent experiments.

compared at each time point. At day 5, $Nrf2^{-/-}$ iDCs proliferate more than $Nrf2^{+/+}$ iDCs at most cell densities (10, 25, 50, 75, and 100 x 10^3 cells/ml, **Figure 3.1*A***). However on day 6, $Nrf2^{-/-}$ iDCs proliferate more than $Nrf2^{+/+}$ iDCs only at 10 x 10^3 cells/ml while other cell densities showed no differences as shown in **Figure 3.1*B***. This difference continues on day 7 at the same cell density (10 x 10^3 cells/ml) but other cell densities showed opposite effects where $Nrf2^{+/+}$ iDCs proliferate more than $Nrf2^{-/-}$ iDCs as in **Figure 3.1*C***. These results demonstrated that, depending on cell density, Nrf2 has the potential to affect DC proliferative capacity.

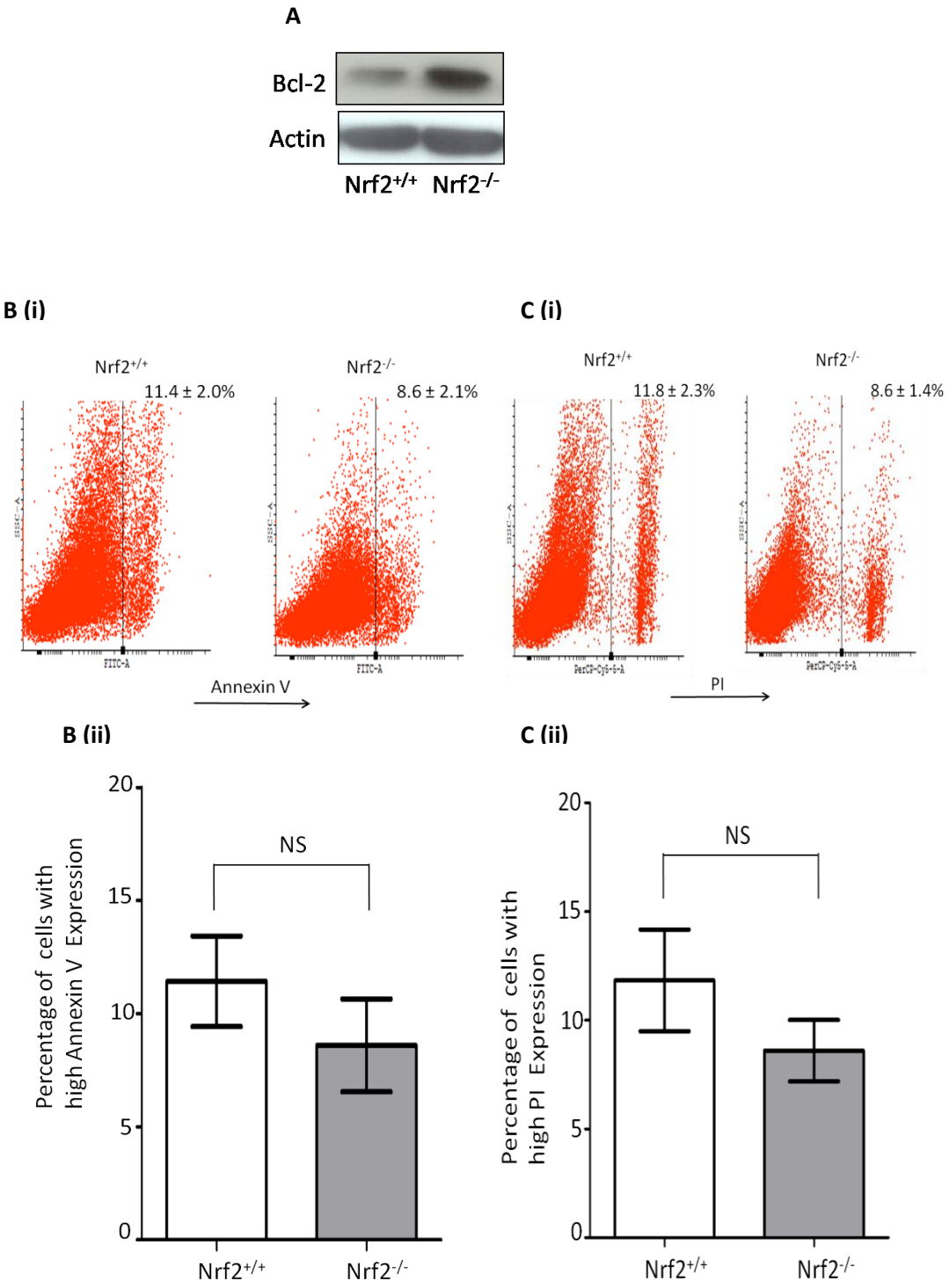
3.2.2 Loss of Nrf2 is associated with increased levels of anti-apoptotic protein, Bcl-2, but does not increase DC survival

Altered proliferative capacity of $Nrf2^{-/-}$ iDCs could also be accompanied by altered survival of these iDCs. The Bcl-2 protein serves as a critical regulator of pathways involved in apoptosis and increased Bcl-2 expression is associated with increased cell survival (Chao and Korsmeyer, 1998). Therefore we sought to examine the level of Bcl-2 by Western immunoblotting. The result revealed an increase in the expression of Bcl-2 in $Nrf2^{-/-}$ iDCs basally in comparison to $Nrf2^{+/+}$ iDCs (**Figure 3.2*A***). In order to test whether increased Bcl-2 expression leads to increased survival (or reduced cell death) of $Nrf2^{-/-}$ DC, we examined cell apoptosis using FITC Annexin V staining which binds to phophatidyl serine (PS) molecules. Phosphatidyl serine is exposed on the inverted phospholipid bilayer cell membrane in one of the early events involved in apoptosis (Fadok et al., 1992; Vermes et al., 1995). While loss of Nrf2 resulted in a reduction in the proportion of Annexin V positive DCs (apoptotic DCs) in relation to the wild type control, this was not statistically significant (**Figure 3.2*B*** 8.6 ± 2.1% in $Nrf2^{-/-}$ iDCs *versus* 11.4 ± 2.0% in $Nrf2^{+/+}$ iDCs, $p > 0.05$).

Since Annexin V staining positivity precedes the loss of membrane integrity (which accompanies the later stages of cell death) resulting from either apoptotic or

necrotic processes, staining with Annexin V-FITC is typically used in conjunction with a live/dead cell-differentiating dye such as propidium iodide (PI) to allow the investigator to identify early apoptotic cells (PI negative, Annexin V-FITC positive) and dead cells (PI positive, AnnexinV-FITC positive).

DC necrosis measured by propidium iodide (PI) staining (Sawai and Domae, 2011) revealed a reduction in the proportion of necrotic cells in $Nrf2^{-/-}$ iDCs when compared with $Nrf2^{+/+}$ iDCs, but again this was not statistically significant (**Figure 3.2*C*** 8.6 ± 1.4% in $Nrf2^{-/-}$ iDCs *versus* 11.8 ± 2.3% in $Nrf2^{+/+}$ iDCs, $p > 0.05$). To test how these DCs respond to the apoptosis inducing drug e.g. dexamethasone (Abe and Thomson, 2006; Cifone et al., 1999), $Nrf2^{-/-}$ and $Nrf2^{+/+}$ iDCs were treated with 1 µM dexamethasone for 18 h and cell apoptosis and necrosis were measured. Results revealed that dexamethasone induced a significant increase in cell apoptosis and necrosis in both genotypes as shown in **Figure 3.2*D*** for apoptosis ($Nrf2^{-/-}$ iDCs 8.6 ± 2.5% in untreated and 20.7 ± 1.4% in dexamethasone treated DCs while in $Nrf2^{+/+}$ iDCs 13.8 ± 2.1% in untreated and 23.2 ± 2.9% in dexamethasone treated DCs). The same apply for cell necrosis **as in Figure 3.2*E*** ($Nrf2^{-/-}$ iDCs 8.4 ± 1.3% in untreated and 17.8 ± 1.8% in dexamethasone treated DCs while in $Nrf2^{+/+}$ iDCs 11.8 ± 1.9% in untreated and 17.3 ± 1.7% in dexamethasone treated DCs).


A
Bcl-2
Actin
Nrf2+/+ Nrf2-/-
B (i)
C (i)
Nrf2+/+
11.4 ± 2.0%
Nrf2-/-
8.6 ± 2.1%
Nrf2+/+
11.8 ± 2.3%
Nrf2-/-
8.6 ± 1.4%
Annexin V
PI
B (ii)
C (ii)
Percentage of cells with high Annexin V Expression
Percentage of cells with high PI Expression
NS
NS
20
15
10
5
0
Nrf2+/+
Nrf2-/-

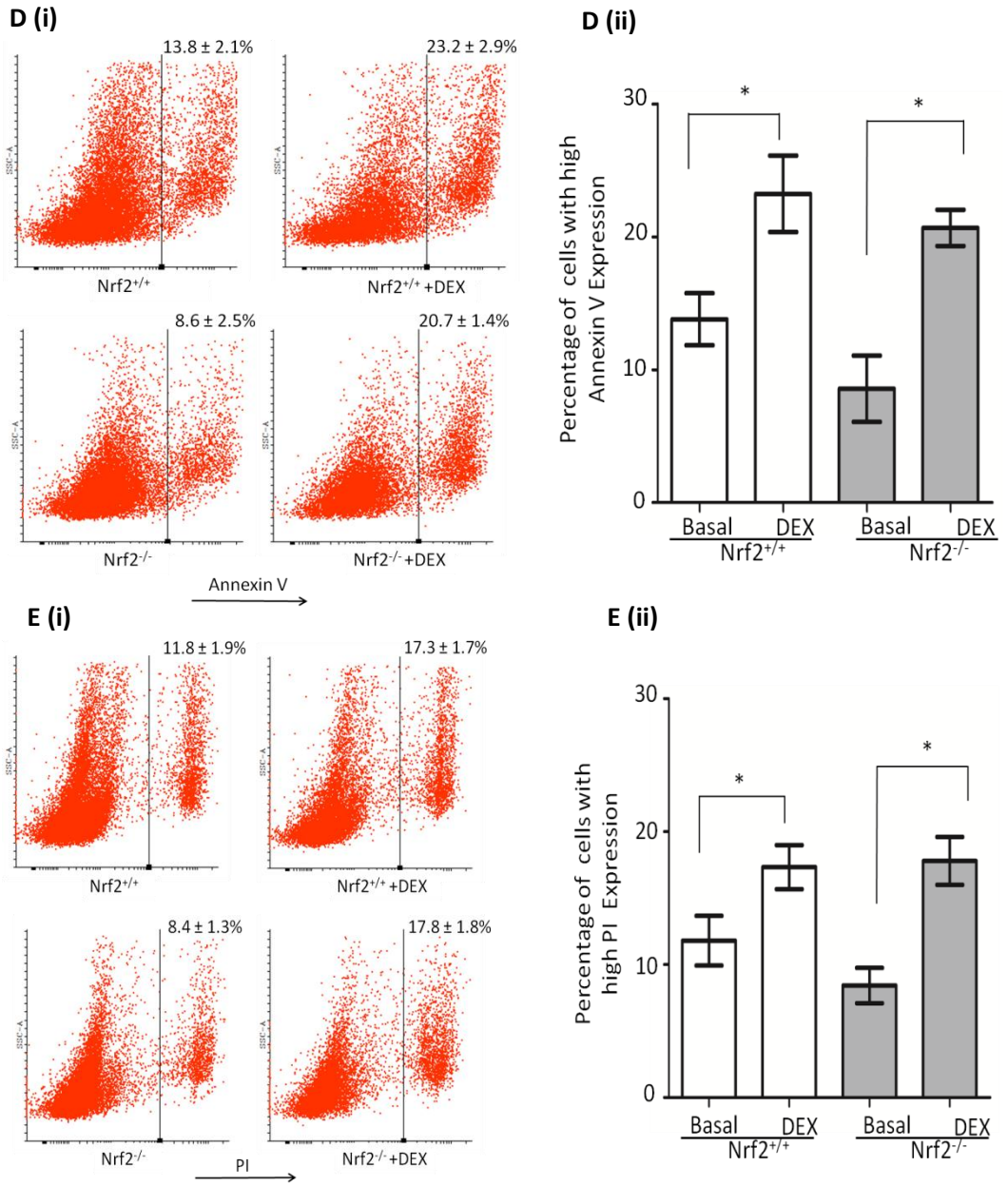


Figure 3.2. Loss of Nrf2 is associated with increased Bcl-2 expression but not DC survival. A, Whole cell lysates from $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs were subjected to SDS-PAGE. Western immunoblotting was used to determine the levels of Bcl-2. Actin was assessed for equal loading of lanes. Data are representative of four independent experiments. On day 7, $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs were stained with Annexin V^{FITC} (**B**) and $PI^{PE\text{-}Cy®5.5}$ (**C**) and analysed by flow cytometry. Percentages of Annexin V^{+} and PI^{+} iDCs are indicated as percentage represented (**Bi**) and (**Ci**) and quantified in (**Bii**) and (**Cii**). On day 6, $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs were treated with dexamethasone (DEX) 1µM for 18 h or left untreated (basal) and then cells were stained with Annexin V^{FITC} (**D**) and $PI^{PE\text{-}Cy®5.5}$ (**E**) and analysed by flow cytometry. Percentages of Annexin V^{+} and PI^{+} iDCs are indicated as percentage represented

(**Di**) and (**Ei**) and quantified in (**Dii**) and (**Eii**). Data are representative of 4 independent experiments. (*, $p < 0.05$; NS- NS-Not Significant).

3.2.3 Loss of Nrf2 results in increased ROS levels without increase in ATP production in immature DCs

Nrf2 signalling increases transcription of antioxidant genes that counter elevations in intracellular ROS (Reddy et al., 2007b). The maintenance of DC intracellular redox status is imperative for appropriate DC immune functioning (Williams et al., 2008). However, the potential effect caused by the loss of Nrf2 on DC ROS levels has not yet been clearly defined. We therefore examined ROS levels using the fluorescent ROS indicator, dihydroethidium, in $Nrf2^{-/-}$ and $Nrf2^{+/+}$ iDCs basally and in response to the oxidative stress inducer, hydrogen peroxide (H_2O_2) and the TLR agonist, LPS. As demonstrated in **Figure 3.3*A***, $Nrf2^{-/-}$ iDCs had significantly higher basal ROS levels in comparison with $Nrf2^{+/+}$ iDCs (mean fluorescence intensity 1341 ± 98 *versus* 833 ± 112, $p < 0.05$). Furthermore, $Nrf2^{-/-}$ iDC ROS levels were significantly increased in comparison with their wild type counterpart following treatment with H_2O_2 (mean fluorescence intensity 2159 ± 116 *versus* 1482 ± 102, $p < 0.05$) and LPS (mean fluorescence intensity 1557 ± 137 *versus* 1026 ± 102, $p < 0.05$). Increased ROS levels may result from enhanced cell oxidative phosphorylation due to increase adenosine triphosphate (ATP) production (Del Prete et al., 2008; Minet and Gaster, 2012). To test this possibility, we measured ATP production by DCs. Results show that $Nrf2^{-/-}$ iDCs produce more ATP than $Nrf2^{+/+}$ iDCs, but this was not statistically significant as shown in **Figure 3.3*B*** (251 ± 39 x 10^3 *versus* 210 ± 26.5 x 10^3, $p > 0.05$).

Taken together, these results suggest that loss of Nrf2 impairs the capacity of iDCs to maintain redox homeostasis.

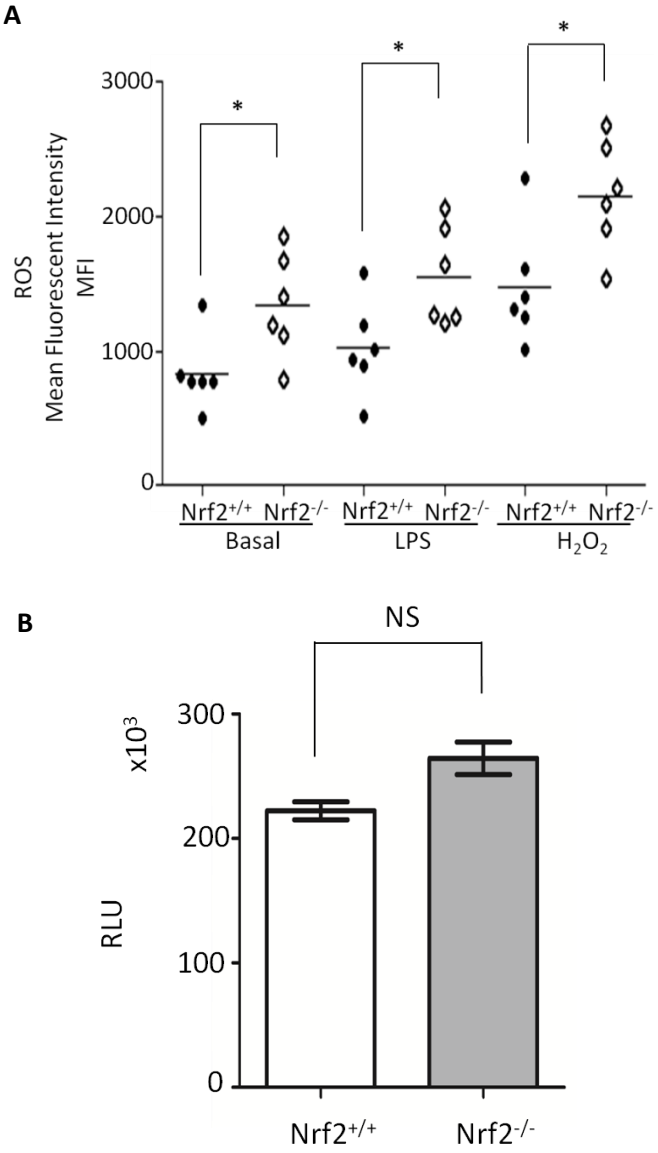


Figure 3.3. Loss of Nrf2 results in increased DC intracellular ROS Levels but is not accompanied by increased oxidative metabolism. A, $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs were untreated or treated with LPS (1 µg/ml) or H_2O_2 (1 mM) for 10 minutes at 37°C. Cells were then incubated with the ROS-indicator DHE before analysis by flow cytometry. Data-points represent mean fluorescence intensity of six separate measurements from 2 independent experiments. Horizontal line represents mean. Statistical significance was tested by unpaired Student's t-test (*, $p<0.05$). **B,** $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs harvested and replated at $30x10^3$ cell/ 100 µl/ well in 96-well flat-bottom plate and ATP was measured using a luminescence assay expressed as Relative luminescence unit (RLU). Statistical significance was tested by unpaired Student's t-test (NS- NS-Not Significant). Data are representative of three independent experiments.

3.2.4 Reduction of cellular glutathione levels leads to increased ROS

Nrf2 is a critical biosynthetic regulator of the antioxidant glutathione (GSH) (Reddy et al., 2007a). Our lab has shown that Nrf2-deficient DCs exhibit diminished GSH levels and reduction of GSH levels by buthionine sulfoximine (BSO) in wild type iDCs did not recapitulate the $Nrf2^{-/-}$ iDCs altered phenotype and function (Aw Yeang et al., 2012). We therefore sought to test a possible link between GSH and ROS, and found that $Nrf2^{+/+}$ iDCs treated with BSO exhibited high levels of ROS that are comparable with $Nrf2^{-/-}$ iDCs (**Figure 3.4**). This further supports the assumption that altered DC phenotype and function may potentially be ROS derived.

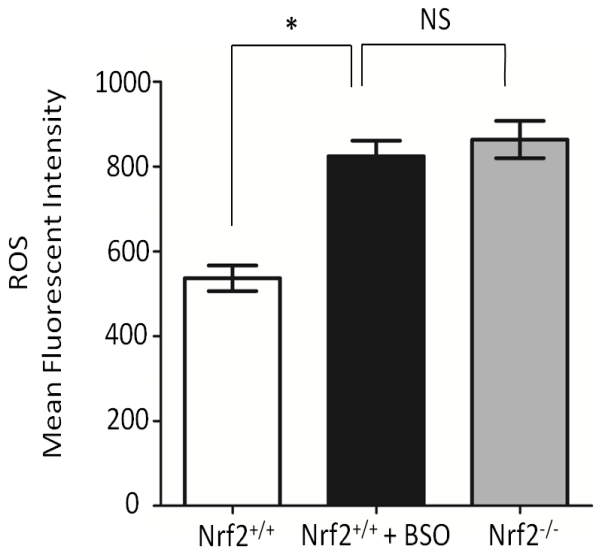
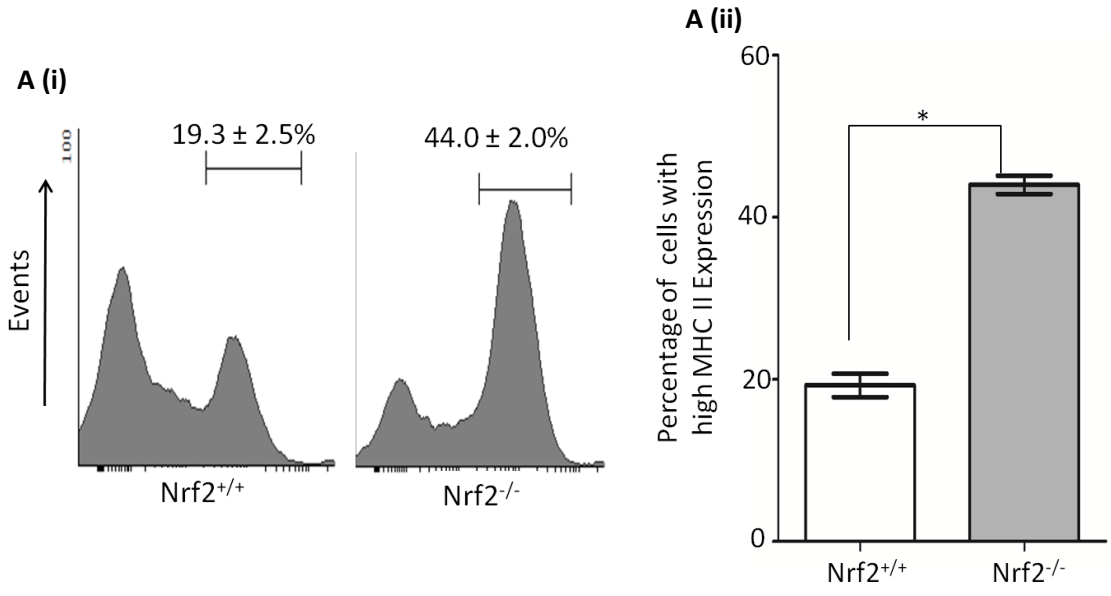


Figure 3.4. Reduction of cellular glutathione leads to increased ROS. $Nrf2^{+/+}$ iDCs untreated or treated with BSO (100 μM) for 24h with $Nrf2^{-/-}$ iDCs used as control. Then cells were incubated with the ROS indicator dihydroethidium and analysed by flow cytometry. Data are presented as average mean fluorescence intensity ± S.D. Statistical significance was assessed using unpaired Student's *t* test. Data are representative of three independent experiments. (*, $p<0.05$; NS-Not Significant).

3.2.5 Loss of Nrf2 results in altered iDCs phenotype

Elevated ROS levels have been shown to enhance DC co-stimulatory molecule expression and maturation (Kantengwa et al., 2003; Matsue et al., 2003b). To evaluate the role of Nrf2 in regulating the phenotype of iDCs, we compared the maturation states of $Nrf2^{-/-}$ and $Nrf2^{+/+}$ iDCs. Maturation is accompanied by an increase in the proportion of DCs that express higher levels of co-stimulatory molecules (Steinman, 2003). Immature DCs are composed of subpopulations that express either high or low levels of co-stimulatory molecules (**Figure 3.5*A***). Flow cytometric analysis of iDCs demonstrated that the proportion of iDCs that express high levels of MHC class II, CD86, and CD40 was significantly increased in the $Nrf2^{-/-}$ iDCs in comparison with the $Nrf2^{+/+}$ iDCs as shown in **Figure 3.5*A*** for MHC class II ($Nrf2^{-/-}$ 44.0 ± 2.0% *versus* $Nrf2^{+/+}$ 19.3 ± 2.5%, $p < 0.05$); **3.5*B*** for CD86 ($Nrf2^{-/-}$ 37.1 ± 2.8% *versus* $Nrf2^{+/+}$ 17.2 ± 1.0%, $p < 0.05$); and **3.5*C*** for CD40 ($Nrf2^{-/-}$ 31.9 ± 3.9% *versus* $Nrf2^{+/+}$ 19.7 ± 1.4%, $p < 0.05$).

These results showed that loss of Nrf2 enhances co-stimulatory molecule expression in iDCs



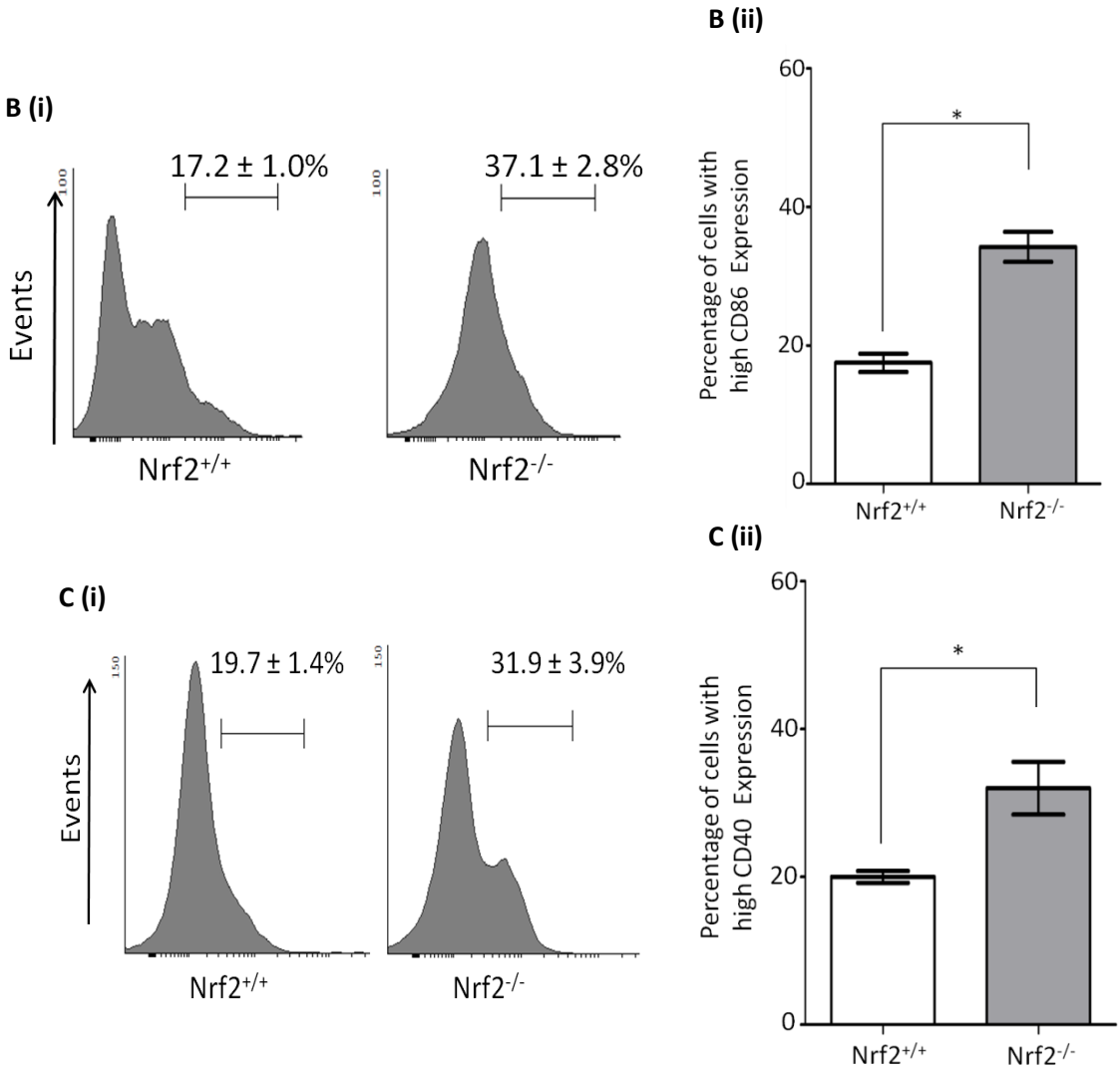
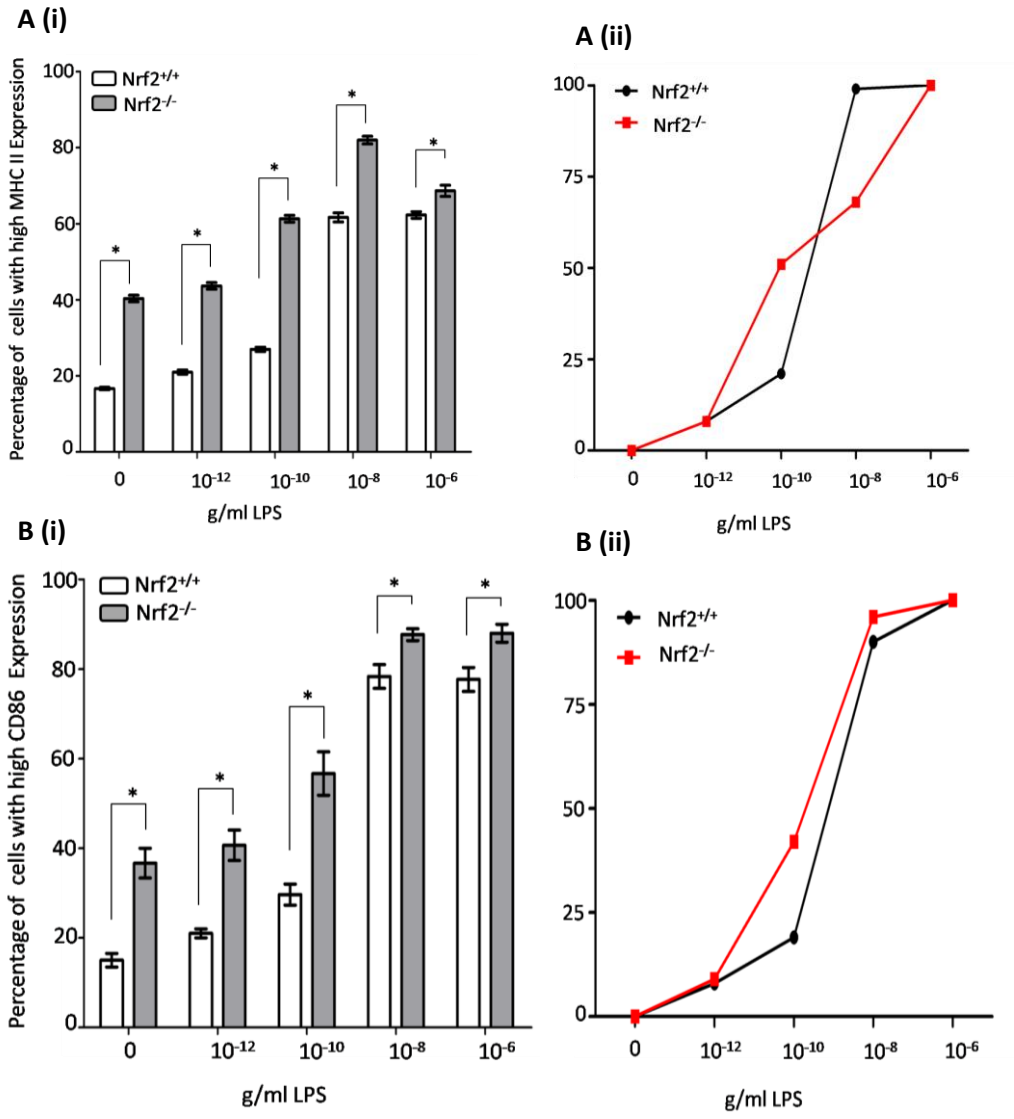


Figure 3.5. Loss of Nrf2 results in enhanced co-stimulatory molecule expression in immature DCs. $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs labelled with antibodies against (**A**) MHC class II, (**B**) CD86 and (**C**) CD40. Co-stimulatory receptor expression was determined by flow cytometry and presented as representative histograms and percentage of cells expressing high MHC class II, CD86 or CD40 indicated above the marker with Bar charts for the percentages of cells expression high MHC class II (**Aii**), CD86 (**Bii**) and CD40 (**Cii**). Data derived from three independent experiments are presented as average percentage ± SD. Statistical significance was assessed using unpaired Student's *t* test (*, $p<0.05$)**.**

3.2.6 Loss of Nrf2 does not alter LPS responsiveness of DCs

To test whether loss of Nrf2 could affect the DC responsiveness to LPS stimulation, we examined the effect of LPS-induced maturation in $Nrf2^{+/+}$ and $Nrf2^{-/-}$ DC. We found that DCs from $Nrf2^{-/-}$ show higher expression of MHC class II and CD86 in response to all concentrations of LPS (**Figure 3.6*A panel (i) and* 3.6*B panel (i)***), but for CD40 the higher expression only observed at low concentrations of LPS (**Figure 3.6*C panel (i)***). However, normalising of the raw data reveals a similar degree of responsiveness to LPS in both genotypes for MHC class II (**Figure 3.6A *panel (ii)***), CD86 (**Figure 3.6B *panel (ii)***) and CD40 **Figure 3.6C *panel (ii)***).

These results showed DC responsiveness to LPS is not affected by loss of Nrf2.



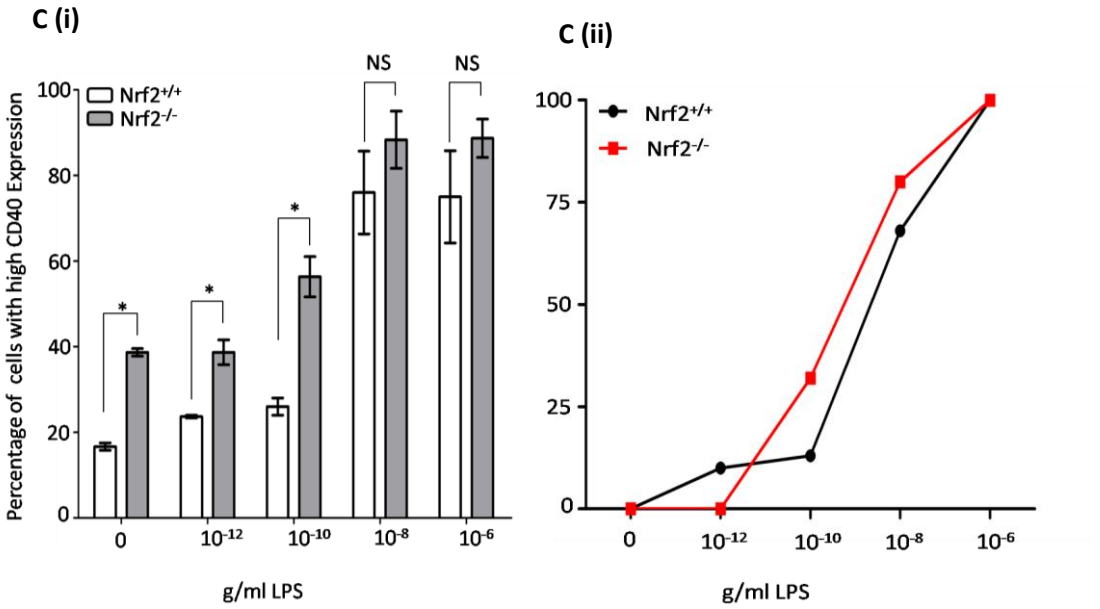


Figure 3.6. Responsiveness of DCs to LPS is unaffected by loss of Nrf2. $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs treated with or without indicated doses of LPS for 18 h. MHC class II (**Ai**), CD86 (**Bi**) and CD40 (**Ci**) expression was determined by flow cytometry and presented as percentage of cells expressing high MHC class II or CD86. Raw data have been normalised for both genotype by subtracting the response percentages of (0 g/ml LPS) from themselves and all the other response percentages so that they have same starting point of 0% and highest percentage of receptor expression exhibited by each genotype was set at 100% maximal response and all other were relative to it as shown in MHC class II (**Aii**), CD86 (**Bii**) and CD40 (**Cii**). Data derived from three independent experiments are presented as average percentage ± SD. Statistical significance was assessed using unpaired Student's *t* test (*, $p<0.05$; NS-Not Significant).

3.2.7 Increased co-stimulatory receptor expression of $Nrf2^{-/-}$ iDCs is associated with enhanced antigen-specific CD8 T cell stimulatory capacity

The response of CD8 cytotoxic T cells to intracellular pathogens and tumours is generally attributed to T cell receptor (TCR)-mediated signalling events triggered by specific interaction of peptide–major histocompatibility complex (MHC) presented

on DCs with the CD8 TCRs (Berg and Forman, 2006; Guermonprez et al., 2002). However, full CD8 T cell activation requires co-stimulatory inputs provided through DC receptors such as CD86 (Chai et al., 1999). Immature DCs expressing low levels of co-stimulatory molecules are unable to stimulate a fully competent antigen-specific CD8 T cell response (Bachmann et al., 1999b). Therefore, maturation of DCs is crucial for the initiation of CD8 cytotoxic T cell dependent immunity. Given that $Nrf2^{-/-}$ iDCs have elevated expression of CD86, CD40, and MHC class II, we postulated that these iDCs would be capable of inducing an antigen-specific CD8 T cell response. To address this, we utilized a TCR transgenic mouse model wherein the CD8 T cells express a T cell receptor (F5 TCR) that recognizes an antigenic peptide, NP68, when presented by DCs (Mamalaki et al., 1992). The functional consequence of changes in DC co-stimulatory molecule expression can be assessed by the ability of NP68-bearing DCs to stimulate F5 CD8 T cell proliferation.

$Nrf2^{+/+}$ iDCs pulsed with increasing concentrations of NP68 did not induce specific F5 CD8 T cell proliferation until 10 nM NP68 (**Figure 3.7**), with a greater proliferation seen at higher concentrations of NP68 (100 nM). In contrast, $Nrf2^{-/-}$ iDCs were able to induce significant F5 CD8 T cell proliferation even at 1 nM NP68 and a 3-to-10-fold higher degree of T cell proliferation when compared with $Nrf2^{+/+}$ iDCs at all NP68 concentrations (**Figure 3.7**, $p < 0.05$).

These data demonstrate that the enhanced maturation phenotype of $Nrf2^{-/-}$ iDC is associated with an increased capacity to induce antigen-specific CD8 T cell stimulation.

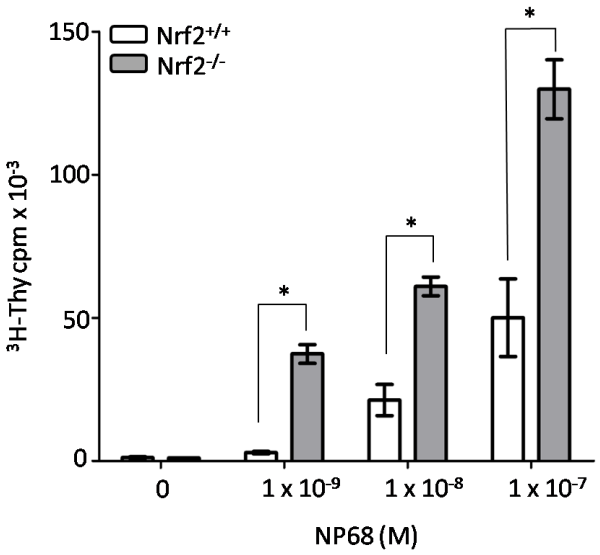


Figure 3.7. Increased T cell stimulatory capacities of Nrf2$^{-/-}$ iDCs. Nrf2$^{+/+}$ and Nrf2$^{-/-}$ iDCs were pulsed with increasing concentrations of NP68 antigenic peptide, and DCs were then co-cultured with F5 CD8 T cells for 72 h.[^{3}H]Thymidine (^{3}H-Thy) was added for the last 16 h. Proliferation of T cells was determined by scintillation counting of incorporated [^{3}H] Thymidine. Data are presented as average [^{3}H]Thymidine scintillation counts ± S.D. Statistical significance was tested by one-way analysis of variance (*, $p<0.05$). Data are derived from three independent experiments.

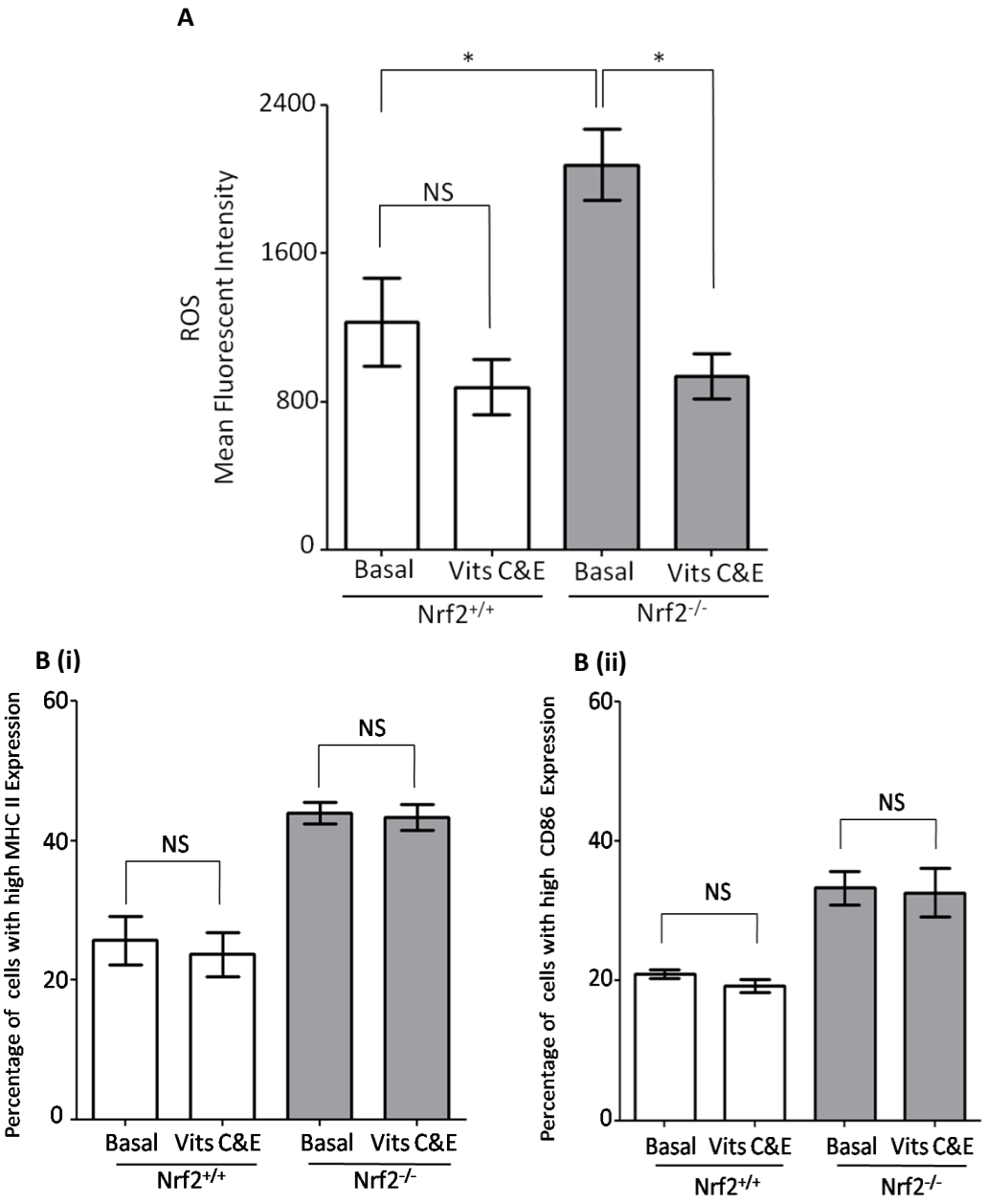
3.2.8 Altered immature DC function due to the loss of Nrf2 is not dependent on elevated ROS

Loss of Nrf2 in iDCs leads to elevated ROS levels (**Figure 3.3*A***), increased co-stimulatory molecule expression (**Figure 3.5*A***), and enhanced T cell stimulatory potential (**Figure 3.7**) (Aw Yeang et al., 2012). We investigated whether the elevated ROS contributed to increased co-stimulatory molecule expression by reducing ROS to normal levels using antioxidants in these cells. Vitamins C and E possess antioxidant activity and are known to reduce ROS levels (Jeong et al., 2011;

Roche et al., 2009). $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs were treated with vitamins C and E for 48 h, and ROS levels measured by flow cytometry. A significant reduction in ROS levels was observed in vitamin-treated $Nrf2^{-/-}$ iDCs compared with untreated controls (**Figure 3.8*A***, mean fluorescence intensity, vitamin-treated $Nrf2^{-/-}$ iDCs 2079 ± 132 *versus* untreated $Nrf2^{-/-}$ iDCs 938 ± 213, $p < 0.05$). A slight reduction in the ROS levels was also seen in vitamin-treated $Nrf2^{+/+}$ iDCs, which was not statistically significant (**Figure 3.8*A***, mean fluorescence intensity, vitamin-treated $Nrf2^{+/+}$ iDCs 1230 ± 209 *versus* untreated $Nrf2^{+/+}$ iDCs 878 ± 148, $p > 0.05$). It is important to note that the ROS levels in vitamin-treated $Nrf2^{-/-}$ iDCs was equivalent to that in untreated $Nrf2^{+/+}$ iDCs (**Figure 3.8*A***). To test whether elevated intracellular redox levels is responsible for increased DC co-stimulatory molecule expression, we measured MHC class II and CD86 expression in $Nrf2^{-/-}$ iDCs following vitamins treatment. As shown in **Figure 3.8*B***, there was no significant difference in the co-stimulatory molecules expression between untreated controls and vitamins treatment groups in both MHC class II (23.1 ± 4.7% *versus* 21.2 ± 4.2%, $p > 0.05$ in $Nrf2^{+/+}$ iDCs; 43.2 ± 2.1% *versus* 42.6 ± 2.7% $p > 0.05$ in $Nrf2^{-/-}$ iDCs, ***panel (i)***) and CD86 (18.4 ± 1.9% *versus* 17.2 ± 2.3%, $p > 0.05$ in $Nrf2^{+/+}$ iDCs; 34.8 ± 3.5% *versus* 35.2 ± 4.1%, $p > 0.05$ in $Nrf2^{-/-}$ iDCs, ***panel (ii)***). These results indicate that restoring ROS levels in $Nrf2^{-/-}$ DC to $Nrf2^{+/+}$ DC status does not reverse co-stimulatory molecule expression.

We further investigated whether the lack of changes in co-stimulatory molecule expression in $Nrf2^{-/-}$ iDCs upon ROS reset is also reflected in its ability to induce antigen-specific T cell activation. Consistent with our previous findings, lowering of ROS levels by vitamin treatment did not reduce the potential of NP68-bearing $Nrf2^{-/-}$ iDCs to stimulate F5 CD8 T cell proliferation (**Figure 3.8*C***).

These results demonstrate that altered ROS status associated with loss of Nrf2 does not contribute to increased co-stimulatory molecules expression and T cell stimulatory potential of DCs.


A
*
*
NS
2400
1600
800
0
ROS
Mean Fluorescent Intensity
Basal
Vits C&E
Basal
Vits C&E
Nrf2+/+
Nrf2-/-
B (i)
Percentage of cells with high MHC II Expression
60
40
20
0
NS
NS
Basal
Vits C&E
Basal
Vits C&E
Nrf2+/+
Nrf2-/-
B (ii)
Percentage of cells with high CD86 Expression
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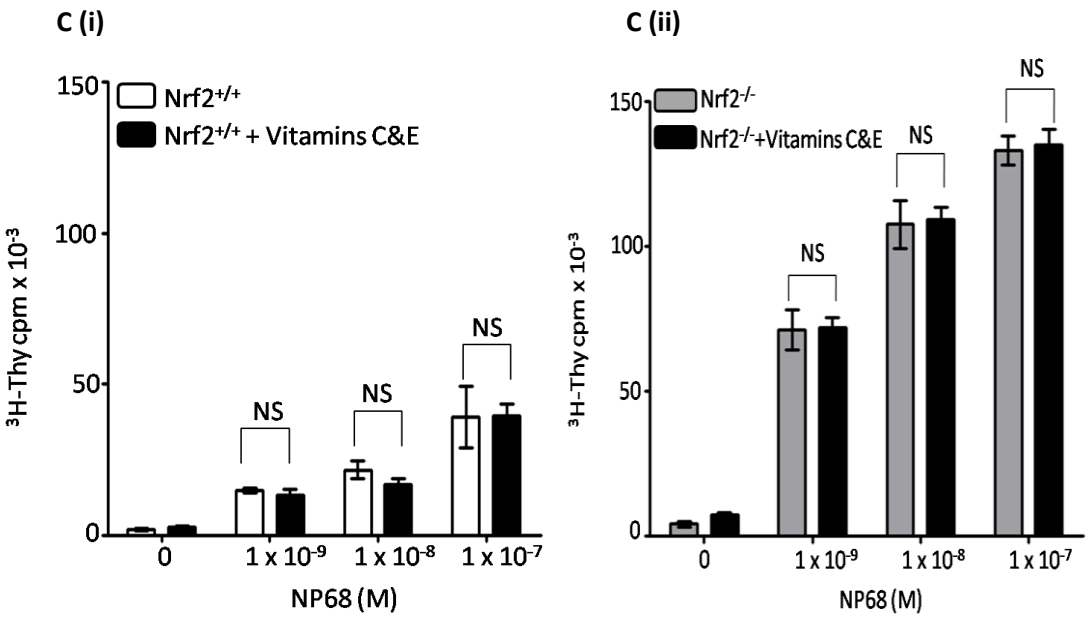


Figure 3.8. Reducing ROS levels does not restore altered phenotype and function of Nrf2-deficient iDCs. A, $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs were treated with or without vitamins C (1 mM) and E (100 µM) for 48 h. then were incubated with the ROS indicator dihydroethidium and analysed by flow cytometry. Data are presented as average mean fluorescence intensity ± S.D. Statistical significance was assessed using unpaired Student's *t* test. Data are representative of three independent experiments. (*, $p<0.05$; NS-Not Significant). **B,** $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs were treated with or without vitamins C (1 mM) and E (100 µM) for 48 h. then were labelled with fluorescent conjugated antibodies against MHC class II and CD86 co-stimulatory molecules. Co-stimulatory molecule expression was determined by flow cytometry. The percentages of iDCs expressing high levels MHC class II (**i**) and CD86 (**ii**) are presented as average percentage ± SD. Statistical significance was assessed using unpaired Student's *t* test. Data are representative of three independent experiments. (NS-Not Significant). Data are derived from three independent experiments. **C,** $Nrf2^{+/+}$ (**i**) and $Nrf2^{-/-}$ (**ii**) iDCs were treated with or without vitamins C (1 mM) and E (100 µM) for 48 h. then were pulsed with increasing concentrations of NP68 antigenic peptide, then co-cultured with F5 CD8 T cells for 72 h. [^{3}H]-Thymidine (^{3}H-Thy) was added for the last 16 h. Proliferation of T cells was determined by scintillation counting of incorporated [^{3}H]-thymidine. Data are presented as average [^{3}H]-thymidine scintillation counts ± S.D. Statistical significance was assessed using one-way ANOVA. Data are representative of three independent experiments. (NS-Not Significant).

3.2.9 The NF-κB pathway does not contribute to altered phenotype and function observed in Nrf2-deficient DC

The transcription factor NF-κB has been implicated in driving transcription of genes for the expression of MHC molecules, co-stimulatory receptors (CD40 and CD86) and cytokines in DCs (Yoshimura et al., 2001). Elevated ROS levels in DCs were found to be associated with alterations in NF-κB signalling (Sheng et al., 2010). Upon activation, upstream IκBα kinase (IKK) complexes are activated, which in turn phosphorylate IκBα, resulting in its ubiquitination and subsequent proteasomal degradation. This facilitates NF-κB translocation into the nucleus, where it can transcribe genes involved in the immune response (Oeckinghaus and Ghosh, 2009). It is unknown whether changes observed in the DC phenotype and function in the absence of Nrf2 was due to alterations in the NF-κB signalling pathway. We therefore first examined the degradation of IκBα upon LPS stimulation in $Nrf2^{+/+}$ iDCs and $Nrf2^{-/-}$ iDCs via western blot. **Figure 3.9*A*** revealed that the kinetics of LPS-triggered IκBα degradation is altered in $Nrf2^{-/-}$ iDCs. While, basally (at 0 min) the levels of IκBα were comparable in both genotypes, upon stimulation with LPS, IκBα degradation could be seen as early as 10 min, progressing to total loss of IκBα at 60 min in $Nrf2^{+/+}$ iDCs, whereas in $Nrf2^{-/-}$ iDCs IκBα degradation was delayed in onset (at 15 min) and incomplete in extent with residual IκBα being detected at 90 min after stimulation. Subsequently, we examined basal and LPS-induced phosphorylation of p65. Although there were no basal differences in phosphorylated NF-κB subunit p65, LPS-induced phosphorylation of p65 was impeded in the $Nrf2^{-/-}$ iDCs when compared with the $Nrf2^{+/+}$ iDCs (**Figure 3.9*B panel (i)***). In $Nrf2^{+/+}$ iDCs, an increase in p65 phosphorylation could be detected at 10 min, peaking at 30 min, followed by a decrease to basal levels at 120 min after LPS stimulation (**Figure 3.9*B panel (i)***). In contrast, no increases in p65 phosphorylation were detected in $Nrf2^{-/-}$ iDCs (**Figure 3.9*B panel (i)***) at any of the time points.

Densitometry analysis of p65 phosphorylation normalised to total p65 protein expression is depicted in **Figure 3.9*B panel (ii)***.

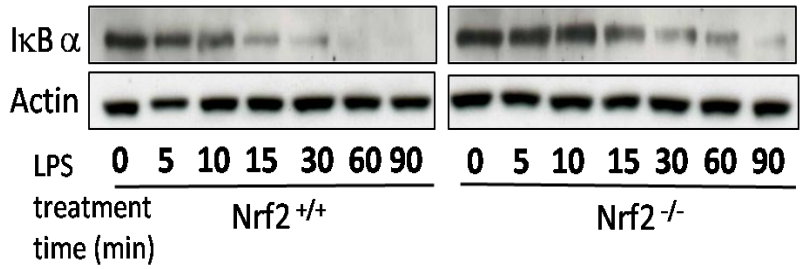
Since there was no change in the basal phosphorylation of p65, we next examined the basal nuclear NF-κB (p65) activity by chemiluminescent transcription factor assay and demonstrated that there were no significant basal differences in NF-κB activity between $Nrf2^{-/-}$ and $Nrf2^{+/+}$ iDCs (**Figure 3.9*C***).

To investigate whether inhibition of NF-κB could reverse the enhanced co-stimulatory molecule expression in $Nrf2^{-/-}$ iDCs, we treated $Nrf2^{-/-}$ and $Nrf2^{+/+}$ iDCs with the p65 inhibitor, Bay 11-7082 (Mori et al., 2002) and assessed its effect on iDC co-stimulatory molecule expression. As expected, p65 inhibition did not reverse the enhanced expression of MHC class II and CD86 in $Nrf2^{-/-}$ iDCs as shown in **Figure 3.9*D panel (i)* and *(ii)*** (MHC class II 40.8 ± 1.6% *versus* 43.8 ± 1.4%, $p > 0.05$; CD86 30.3 ± 0.6% *versus* 33.3 ± 5.5%, $p > 0.05$) and also no changes were observed in treated $Nrf2^{+/+}$ iDCs (MHC class II 13.1 ± 0.2% *versus* 12.3 ± 1.4%, $p > 0.05$; CD86 9.7 ± 0.6% *versus* 11.0 ± 2.6%, $p > 0.05$).

Additionally, the iDC-mediated antigen-specific CD8 T cell proliferation also remained unaltered following p65 inhibition in $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs (**Figure 3.9*E (i) and (ii)***).

Taken together, these results suggest that although loss Nrf2 in iDCs may affect upstream elements of the NF-κB pathway (phosphorylation of p65 and IκBα degradation), it did not affect basal NF-κB activity and thus the enhanced co-stimulatory molecule expression exhibited in $Nrf2^{-/-}$ iDCs is not dependent on NF-κB activity.

A


IκB α
Actin
LPS
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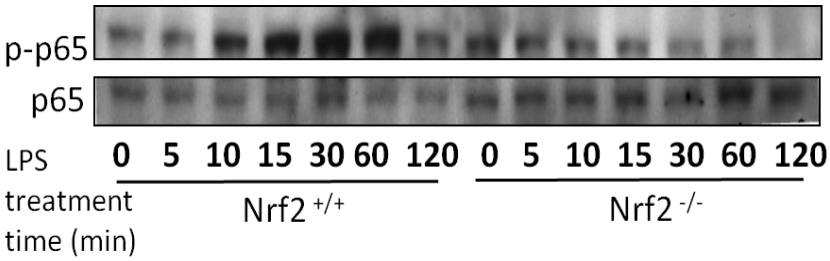
0 5 10 15 30 60 90
treatment
time (min)
Nrf2 +/+
Nrf2 -/-

B (i)


p-p65
p65
LPS
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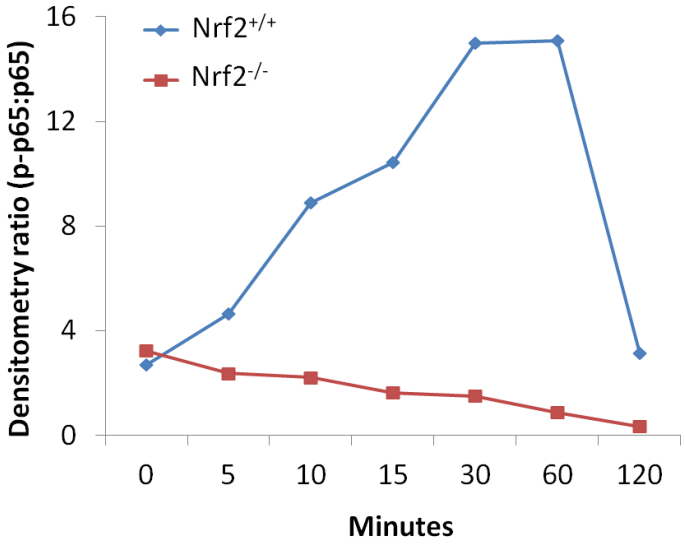
0 5 10 15 30 60 120
treatment
time (min)
Nrf2 +/+
Nrf2 -/-

B (ii)


Nrf2+/+
Nrf2-/-
Densitometry ratio (p-p65:p65)
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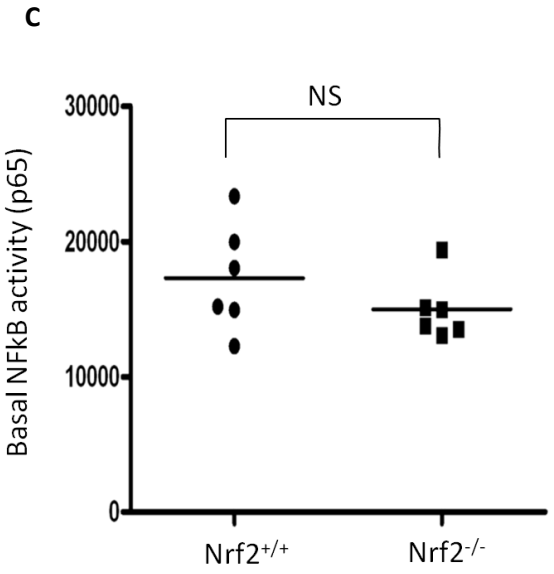
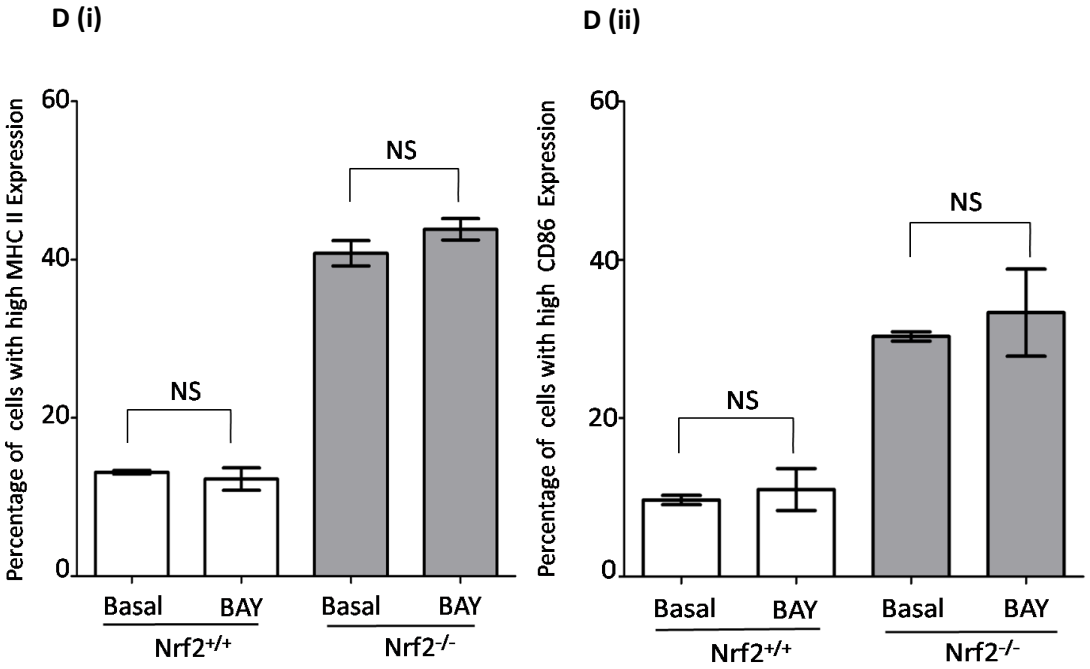
12
8
4
0
0 5 10 15 30 60 120
Minutes


C
NS
30000
20000
10000
0
Basal NFkB activity (p65)
Nrf2+/+
Nrf2-/-


D (i)
Percentage of cells with high MHC II Expression
60
40
20
0
NS
NS
Basal
BAY
Basal
BAY
Nrf2+/+
Nrf2-/-
D (ii)
Percentage of cells with high CD86 Expression
60
40
20
0
NS
NS
Basal
BAY
Basal
BAY
Nrf2+/+
Nrf2-/-

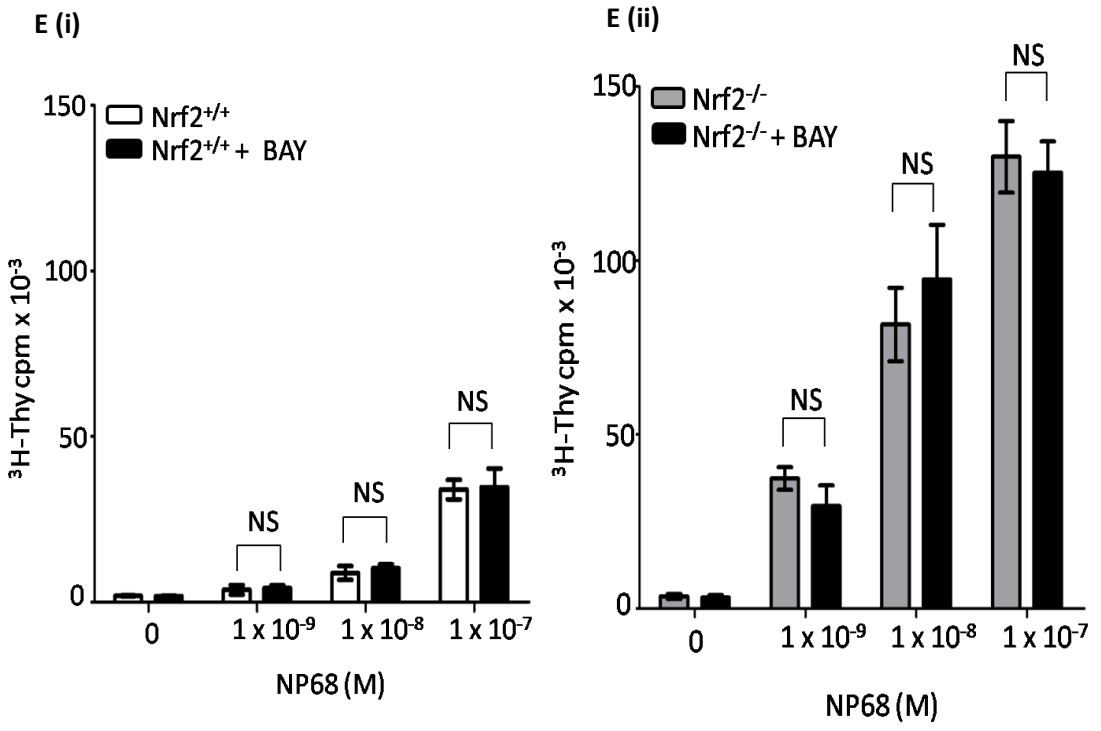


Figure 3.9. The NF-κB pathway does not contribute to altered phenotype and function of Nrf2-deficient iDCs. A and **B,** $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs were treated with LPS (1 μg/ml) for indicated times and whole cell lysates were subjected to SDS-PAGE. The levels of IκBa and actin (**A**) and the phosphorylation levels of p65 and total p65 (**B i**) were assessed by Western blotting and p-p65 protein bands were blotted by densitometry against total p65 (**B ii**). Data are representative of 3 independent experiments. **C,** NF-κB activity in the nuclear lysates of $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs was measured and presented as dot plots with each dot representing mean RLUs (from triplicates). Data are from 6 independent experiments. Horizontal lines indicate means for each group. **D,** $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs treated with or without 10 μM BAY 11-7082 (BAY) for 48 h. MHC class II (**i**) and CD86 (**ii**) expression was determined by flow cytometry and presented as percentage of cells expressing high MHC class II or CD86. Data derived from three independent experiments are presented as average percentage ± SD. (NS-Not Significant). **E,** $Nrf2^{+/+}$ (**i**) and $Nrf2^{-/-}$ (**ii**) iDCs treated with or without 10 μM BAY 11-7082 (BAY) for 48 h. then were pulsed with increasing concentrations of NP68 antigenic peptide, and co-cultured with F5 CD8 T cells for 72 h. [^{3}H]-Thymidine (^{3}H-Thy) was added for the last 16 h. Proliferation of T cells was determined by scintillation counting of incorporated [^{3}H]-thymidine. Data are presented as average [^{3}H]-thymidine scintillation counts ± S.D. Statistical significance was assessed using unpaired Student's t test or one-way ANOVA. Data are representative of three independent experiments. (NS-Not Significant).

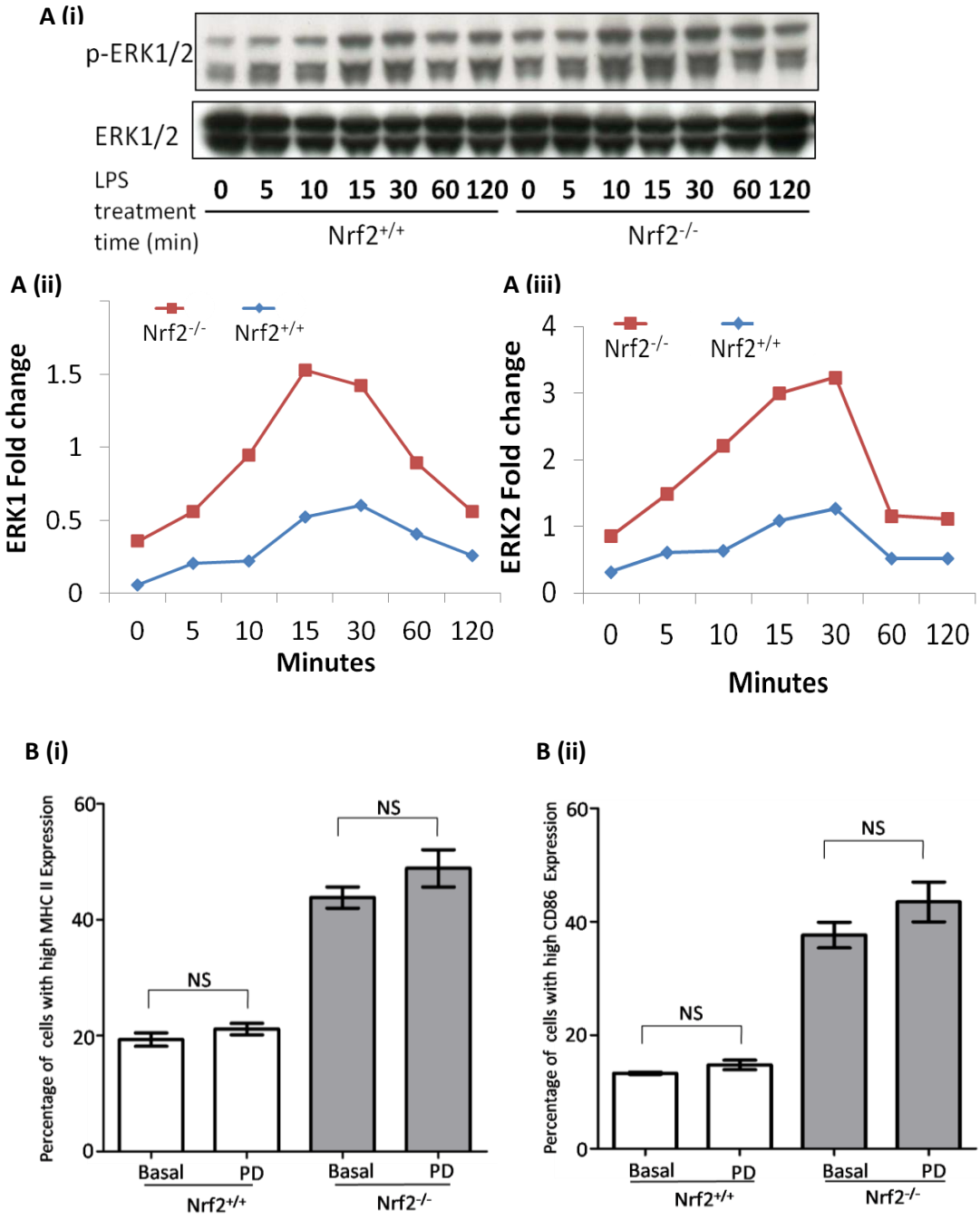
3.2.10 ERK1/2 MAPK is not involved in mediating Nrf2-dependent modulation of DC function

Redox alterations in DCs have been found to be associated with changes in ERK1/2 MAPK signalling pathway (Aiba et al., 2003). Activation of MAPK pathways by stimuli such as LPS results in phosphorylation of ERK1/2 (Rescigno et al., 1999). We therefore investigated whether loss of Nrf2 in DCs affected ERK1/2 signalling. In order to test that we examined the phosphorylation levels of ERK1/2 via Western blot. Results revealed that the level of phosphorylated ERK1/2 was slightly higher in $Nrf2^{-/-}$ iDCs in comparison with $Nrf2^{+/+}$ iDCs both basally and upon LPS stimulation (**Figure 3.10*A* *panel (i)***). Furthermore, $Nrf2^{-/-}$ iDCs exhibited enhanced phosphorylation kinetics of ERK1/2 in response to LPS in comparison with their wild type counterpart. The onset of LPS-induced phosphorylation of ERK1/2 was observed at 10 min in $Nrf2^{-/-}$ iDCs when compared with 15 min for $Nrf2^{+/+}$ iDCs. These changes were more obvious when observed in densitometry analysis of phosphorylated ERK1 normalised to total ERK1 protein expression (**3.10*A* *panel (ii)***) and phosphorylated ERK2 normalised to total ERK2 protein expression (**3.10*A* *panel (iii)***).

To assess whether this change in basal ERK1/2 phosphorylation contributes to the enhanced co-stimulatory molecule expression in $Nrf2^{-/-}$ iDCs, we treated $Nrf2^{-/-}$ and $Nrf2^{+/+}$ iDCs with the ERK inhibitor, PD98059 (Yanagawa et al., 2002), and assessed its effect on iDC co-stimulatory molecule expression. ERK inhibition did not reverse the enhanced expression of MHC class II and CD86 in $Nrf2^{-/-}$ iDCs but rather caused a slight but insignificant increase (MHC class II 43.8 ± 1.8% *versus* 48.9 ± 3.2%, $p > 0.05$; CD86 37.7 ± 2.3% *versus* 43.5 ± 3.5%, $p > 0.05$) (**Figure 3.10*B* *panel (i)*** and ***(ii)***). As reported previously for human DCs (Aiba et al., 2003), similar findings were found in $Nrf2^{+/+}$ iDCs (MHC class II 19.3 ± 1.2% *versus* 21.1 ± 1.0%, $p > 0.05$; CD86 13.3 ± 0.2% *versus* 14.8 ± 0.9%, $p > 0.05$).

Consistent with the lack in the changes in co-stimulatory molecule expression, inhibition of ERK1/2 MAPK did not result in reduced DC-mediated antigen-specific CD8 T cell proliferation in Nrf2$^{-/-}$ iDCs as shown in **Figure 3.10*C panel (i)*** and ***(ii)***.

From these findings we conclude that enhanced co-stimulatory molecule expression in Nrf2$^{-/-}$ iDCs is not dependent on ERK1/2 activity.



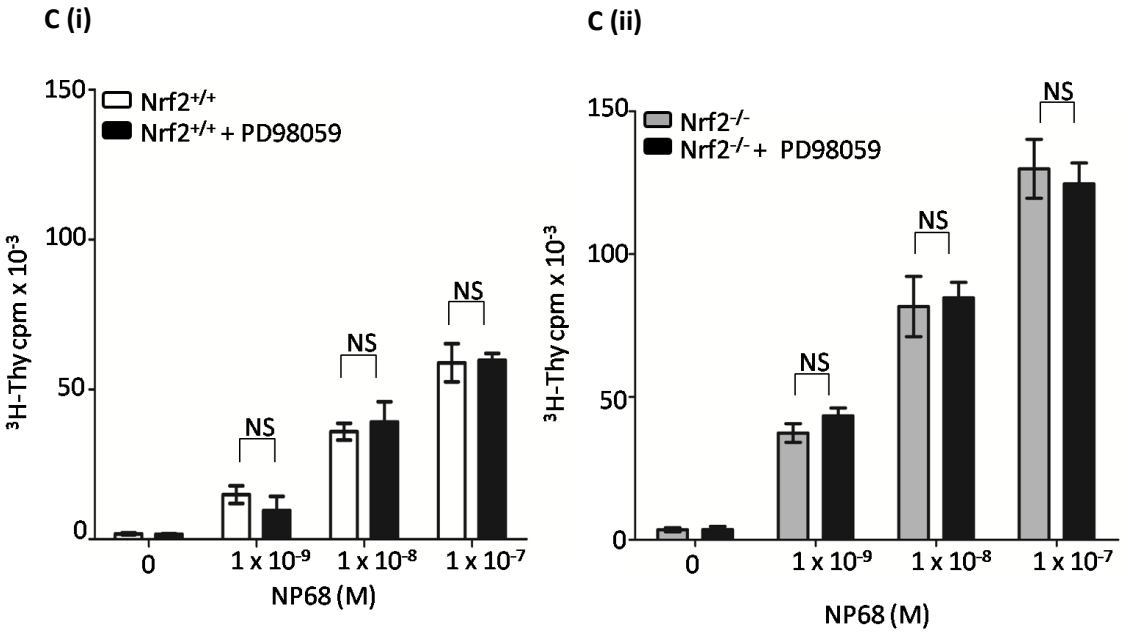


Figure 3.10. ERK1/2 does not contribute to the altered DC phenotype and function in Nrf2^{-}/$^{-}$ iDCs. A, Nrf2$^{+/+}$ and Nrf2$^{-/-}$ iDCs were treated with LPS (1 µg/ml) for indicated time points and whole cell lysates were subjected to SDS-PAGE. The phosphorylation levels of ERK1/2 and total ERK1/2 were assessed by Western blotting **(i)** and p-ERK1 **(ii)** and p-ERK2 **(iii)** protein bands were analysed by densitometry and normalised to total ERK1 and ERK2 respectively. Data are representative of 3 independent experiments. **B,** Nrf2$^{+/+}$ and Nrf2$^{-/-}$ iDCs were treated with or without 50 µM of ERK1/2 inhibitor (PD98059) for 48 h. MHC class II **(i)** and CD86 **(ii)** expression was determined by flow cytometry and presented as percentage of cells expressing high MHC class II or CD86. Data derived from three independent experiments are presented as average percentage ± SD. Statistical significance was assessed using unpaired Student's t test. (NS-Not Significant). **C, (i)** Nrf2$^{+/+}$ and **(ii)** Nrf2$^{-/-}$ iDCs treated with or without 50 µM of ERK1/2 inhibitor (PD98059) for 48 h. then were pulsed with increasing concentrations of NP68 antigenic peptide, and co-cultured with F5 CD8 T cells for 72 h. [^{3}H]-Thymidine (^{3}H-Thy) was added for the last 16 h. Proliferation of T cells was determined by scintillation counting of incorporated [^{3}H]-thymidine. Data are presented as average [^{3}H]-thymidine scintillation counts ± S.D. Statistical significance was assessed using unpaired Student's *t* test or one-way ANOVA. Data are representative of three independent experiments. (NS-Not Significant).

3.2.11 c-Jun N-terminal kinase (JNK) is not involved in mediating Nrf2-dependent modulation of DC function

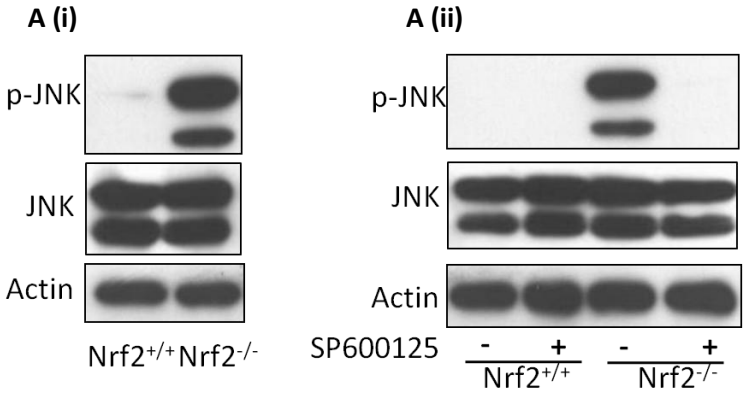
JNK signalling pathway play a vital role in the process of DCs maturation that is distinct from those of the p38MAPK and ERK cascades (Nakahara et al., 2004). It is known to be phosphorylated during the DC maturation process induced by LPS (Arrighi et al., 2001).

In order to examine the contribution of JNK pathway to the altered phenotype and function in Nrf2-deficient DC, we assessed the level of phosphorylated JNK by Western blotting. JNK was found to be basally hyperphosphorylated in $Nrf2^{-/-}$ iDCs compared to $Nrf2^{+/+}$ iDCs **Figure 3.11*A* *panel (i)***, and JNK inhibitor SP600125 inhibited the phosphorylation of JNK in $Nrf2^{-/-}$ iDCs as shown in **Figure 3.11*A* *panel (ii)***.

To verify whether this difference in basal phosphorylation of JNK contributed to the enhanced co-stimulatory molecule expression in $Nrf2^{-/-}$ iDCs, we treated $Nrf2^{-/-}$ and $Nrf2^{+/+}$ iDCs with the JNK inhibitor, SP600125 (Bennett et al., 2001), and assessed its effect on iDCs co-stimulatory molecule expression. JNK inhibition did not reverse the enhanced co-stimulatory molecule expression in $Nrf2^{-/-}$ iDCs to that of the wild type levels (MHC class II 42.8 ± 2.3% *versus* 47.0 ± 7.6%, $p > 0.05$; CD86 44.0 ± 0.6% *versus* 51.8 ± 8.1%, $p > 0.05$ in $Nrf2^{-/-}$ iDCs; and MHC class II 21.3 ± 1.8% *versus* 20.5 ± 1.5%, $p > 0.05$; CD86 20.0 ± 2.1% *versus* 21.0 ± 0.6%, $p > 0.05$ in $Nrf2^{+/+}$ iDCs) as indicated in **Figure 3.11*B* *panel (i)*** and ***panel (ii)***.

Moreover, the iDC-mediated antigen specific CD8 T cell proliferation also remained unaltered following JNK inhibition in $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs (**Figure 3.11*C* *panel (i)*** and ***panel (ii)***).

These findings indicate that JNK is not the main MAPK that is involved in Nrf2-mediated regulation of DC function.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JNK
Actin
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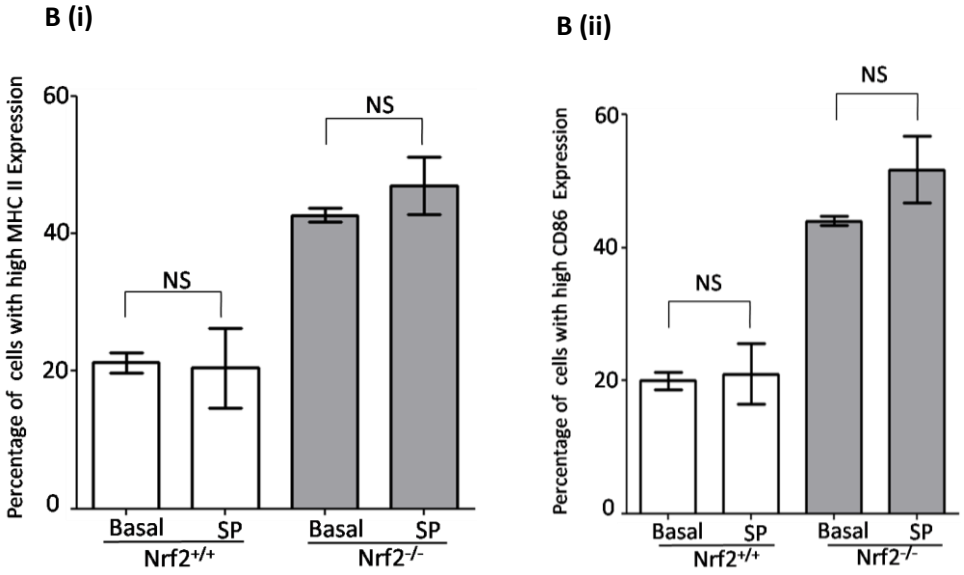
p-JNK
JNK
Actin
SP600125
- + - +
Nrf2+/+
Nrf2-/-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NS
NS
Basal SP Basal SP
Nrf2+/+
Nrf2-/-
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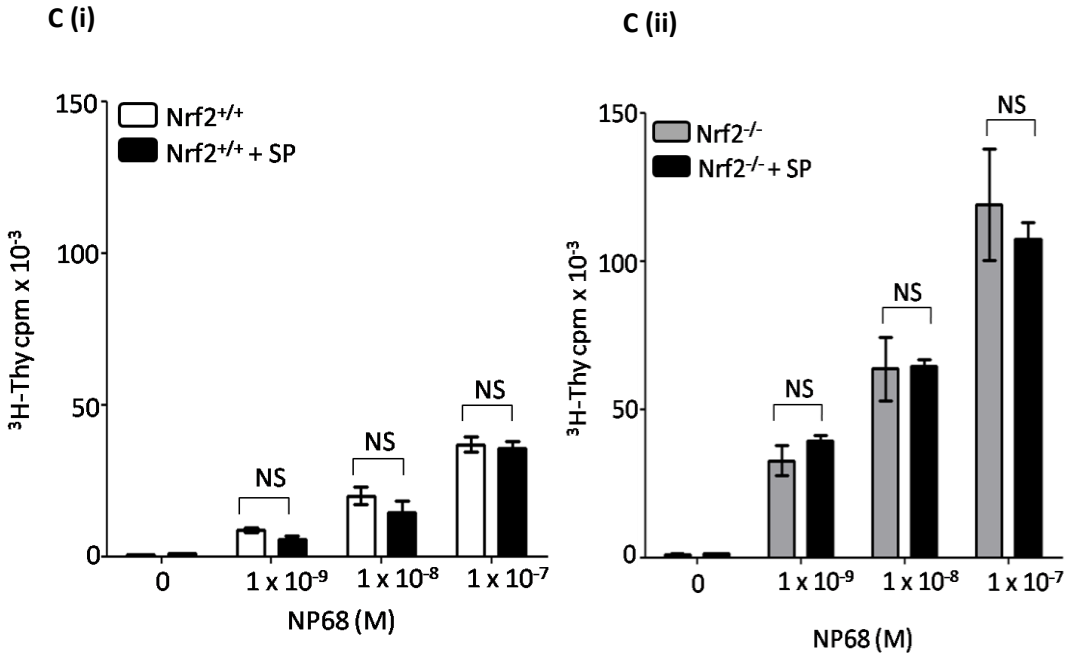


Figure 3.11. Basally activated JNK is not involved in mediating altered phenotype and function in Nrf2- deficient DCs. A (**i**)**,** Whole cell lysates from $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs were subjected to SDS-PAGE. (**ii**), $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs were treated with JNK inhibitor (SP600125) for 1 h or left untreated and whole cell lysates were subjected to SDS-PAGE. The phosphorylation levels of JNK were assessed by Western blotting. Total JNK and actin were assessed as loading control. Data are representative of 3 independent experiments. **B,** $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs treated with or without 10 µM SP600125 (SP) for 48 h. MHC class II (**i**) and CD86 (**ii**) expression was determined by flow cytometry and presented as percentage of cells expressing high MHC class II or CD86. Data derived from three independent experiments are presented as average percentage ± SD. (NS-Not Significant). **C,** $Nrf2^{+/+}$ (**i**) and $Nrf2^{-/-}$ (**ii**) iDCs treated with or without 10 µM SP600125 (SP) for 48 h. then were pulsed with increasing concentrations of NP68 antigenic peptide, and co-cultured with F5 CD8 T cells for 72 h. [^{3}H]-Thymidine (^{3}H-Thy) was added for the last 16 h. Proliferation of T cells was determined by scintillation counting of incorporated [^{3}H]-thymidine. Data are presented as average [^{3}H]-thymidine scintillation counts ± S.D. Statistical significance was assessed using unpaired Student's *t* test or one-way ANOVA. Data are representative of three independent experiments. (NS-Not Significant).

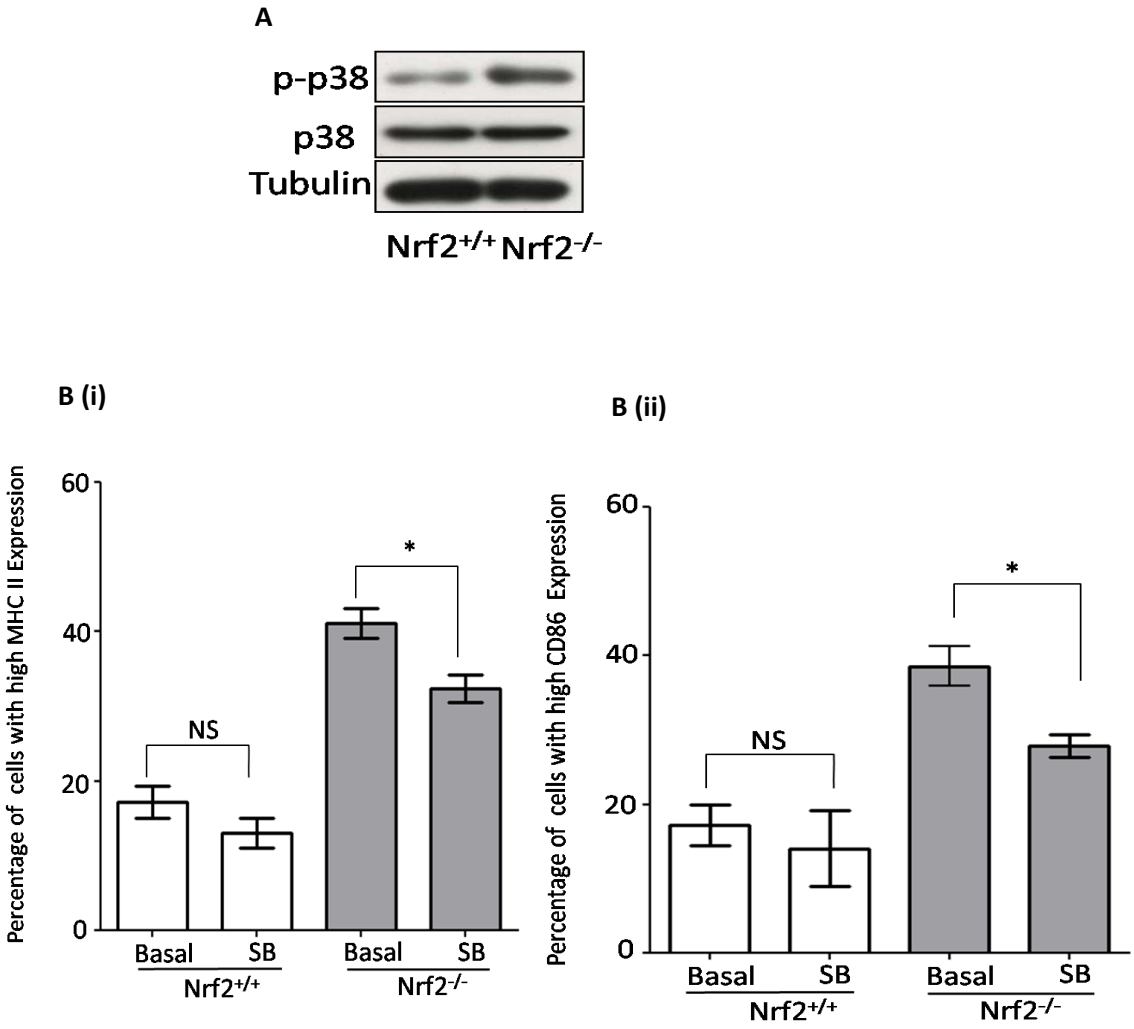
3.2.12 Contribution of p38MAPK activity to Nrf2-dependent regulation of DC immune function

The p38MAPK pathway coordinates DC co-stimulatory molecule expression and DC-mediated T cell activation (Arrighi et al., 2001). As the activity of p38MAPK is regulated by phosphorylation, we assessed the phosphorylation status of p38MAPK. Western blotting analysis revealed marginal differences in p38MAPK phosphorylation between $Nrf2^{-/-}$ and $Nrf2^{+/+}$ iDCs (**Figure 3.12*A***).

Next we further dissected the role of p38MAPK in the altered phenotype and function observed in $Nrf2^{-/-}$ iDCs using pharmacological approaches. Inhibition of p38MAPK activity with SB203580 (English and Cobb, 2002) caused a significant reduction in co-stimulatory molecule expression in $Nrf2^{-/-}$ iDCs (**Figure 3.12*B panel (i)*** and ***panel (ii)***) (MHC class II 41.0 ± 2.5% *versus* 32.3 ± 3.8%, $p < 0.05$; CD86 38.6 ± 3.9% *versus* 27.8 ± 1.2%, $p < 0.05$). Inhibition of p38MAPK in $Nrf2^{+/+}$ iDCs resulted in only a slight, statistically insignificant reduction in co-stimulatory molecule expression (MHC class II 17.2 ± 3.5% *versus* 13.0 ± 2.0%, $p > 0.05$; CD86 17.2 ± 3.2% *versus* 14.0 ± 4.6%, $p > 0.05$).

Consistent with the changes in co-stimulatory molecule expression, inhibition of p38MAPK resulted in significant reductions in DC-mediated antigen-specific CD8 T cell proliferation in $Nrf2^{-/-}$ iDCs with less pronounced effects on $Nrf2^{+/+}$ iDCs (**Figure 3.12*C panel (i)*** and ***panel (ii)***).

These observations suggest that p38MAPK plays a role in the Nrf2-dependent modulation of DC immune functions.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Percentage of cells with high MHC II Expression
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20
0
NS
*
Basal
SB
Basal
SB
Nrf2+/+
Nrf2-/-
B (ii)
Percentage of cells with high CD86 Expression
60
40
20
0
NS
*
Basal
SB
Basal
SB
Nrf2+/+
Nrf2-/-

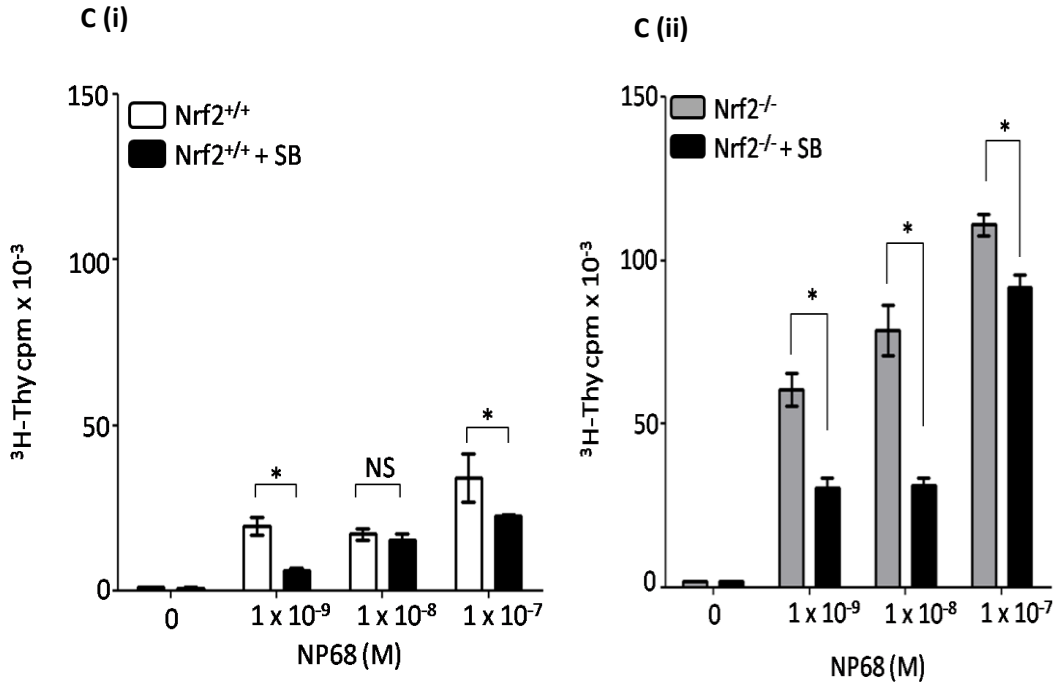


Figure 3.12. Requirement for p38MAPK activity in Nrf2-dependent regulation of DC phenotype and function. A, Whole cell lysates from $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs were subjected to SDS-PAGE. Western immunoblotting was used to determine the levels of phospho-p38 (p-p38) and total p38 (p38). Tubulin was assessed for equal loading. **B,** $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs treated with or without 20 µM SB203580 (SB) for 48 h. MHC class II (**i**) and CD86 (**ii**) expression was determined by flow cytometry and presented as percentage of cells expressing high MHC class II or CD86. Data derived from three independent experiments are presented as average percentage ± SD. Statistical significance was assessed using unpaired Student's *t* test (*, $p<0.05$; NS-Not Significant). **C,** $Nrf2^{+/+}$ (**i**) and $Nrf2^{-/-}$ (**ii**) iDCs treated with or without 20 µM SB203580 (SB) for 48 h. Then, DCs were pulsed with increasing concentrations of NP68 antigenic peptide, and co-cultured with F5 CD8 T cells for 72 h. [^{3}H]-Thymidine (^{3}H-Thy) was added for the last 16 h. Proliferation of T cells was determined by scintillation counting of incorporated [^{3}H]-thymidine. Data are presented as average [^{3}H]-thymidine scintillation counts ± S.D. Statistical significance was assessed using one-way ANOVA. Data are representative of three independent experiments. (*, $p<0.05$; NS-Not Significant).

3.2.13 Contribution of the p38MAPK-CREB/ATF1 signalling pathway to Nrf2-dependent regulation of DC immune function

Major downstream effectors of p38MAPK are the transcription factors, CREB and ATF1 (Ardeshna et al., 2000). Serine phosphorylation of CREB and ATF1 by upstream MAPKs is required for their activation. As p38MAPK was found to be hyperphosphorylated in $Nrf2^{-/-}$ iDCs (**Figure 3.13*A***), we measured the phosphorylation state of CREB and ATF1. Results revealed that CREB and ATF1 were hyperphosphorylated under basal conditions in $Nrf2^{-/-}$ iDCs (**Figure 3.13*B***). A significant reduction in the phosphorylation of CREB and ATF1 was observed upon inhibiting p38MAPK activity using a pharmacological inhibitor (SB203580) as seen in **Figure 3.13*C***. This suggests that CREB and ATF1 phosphorylation in DCs are dependent on p38MAPK activity. There is evidence in other cell types that in addition to p38MAPK, ERK1/2 can also mediate CREB/ATF1 phosphorylation. We tested the requirement of ERK1/2 for phosphorylation of CREB and ATF1 in the DCs under basal and LPS-stimulated conditions using ERK1/2 inhibitor (PD98059) (Yanagawa et al., 2002). As seen in **Figure 3.13*D***, inhibition of ERK1/2 did not reduce the level of phosphorylated-CREB/ATF1 under basal conditions. LPS stimulation markedly increased the phosphorylation of CREB/ATF1 in $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs (**Figure 3.13*D***) and inhibition of ERK1/2 did not reduce the phosphorylation of CREB/ATF1 under LPS-stimulated conditions in both $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs (**Figure 3.13*D***). Consistent with the previous result (**Figure 3.13*C***), p38MAPK inhibition reduced CREB/ ATF1 phosphorylation levels both basally and upon LPS stimulation. To test whether the basal hyperphosphorylation of CREB/ATF1 in $Nrf2^{-/-}$ iDCs could be the result of elevated ROS (Kim et al., 2012), we treated the DCs with vitamins and showed that decreasing elevated ROS did not reverse the increased CREB/ATF1 phosphorylation (**Figure 3.13*E***).

These results indicate that Nrf2 is required for controlling the activity of the p38MAPK-CREB/ ATF1 signalling axis in DCs.

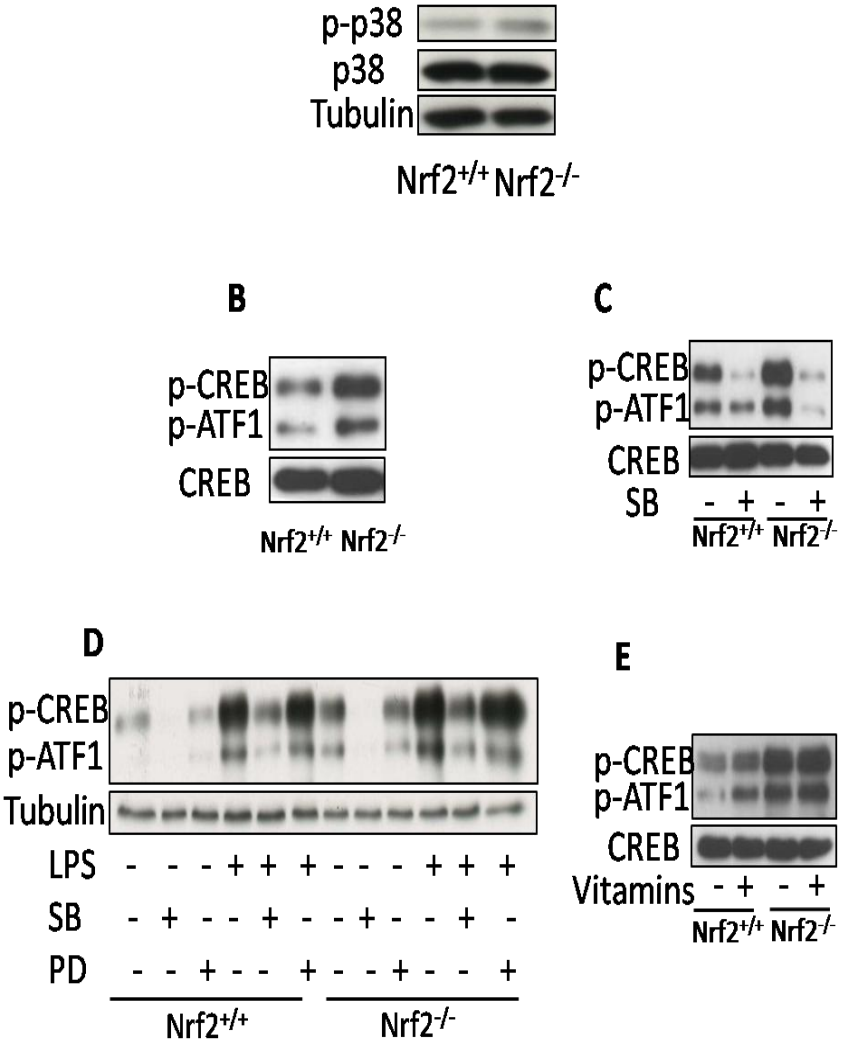


Figure 3.13. Loss of Nrf2 perturbs p38MAPK-CREB/ATF1 signalling in DCs. Whole cell lysates from $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs were subjected to SDS-PAGE. Western immunoblotting was used to determine the levels of **A,** Phospho-p38 (p-p38) and total p38 (p38); **B,** Phospho-CREB (p-CREB), phospho-ATF1 (p-ATF1) and total CREB (CREB). **C,** Whole cell lysates from $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs treated with or without 20 µM of p38 activity inhibitor SB203580 (SB) for 1 h were subjected to SDS-PAGE. Phosphorylation of CREB and ATF1 (p-CREB and p-ATF1) and total CREB (CREB) were assessed. **D,** Whole cell lysates from $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs treated with or without 50 µM of ERK1/2 inhibitor, PD98059 (PD) or 20 µM of SB203580 (SB) for 1 h in the presence or absence of LPS (1 µg/ml) for the last 30 min were subjected to SDS-PAGE. Phosphorylation of CREB and ATF1 (p-CREB and p-ATF1) and tubulin were assessed. **E,** Whole cell lysates from $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs treated with or without vitamins C (1 mM) and E (100 µM) for 48 h were subjected to SDS-PAGE. Phosphorylation of CREB (p-CREB) and ATF1 (p-ATF1) and total CREB (CREB) were assessed. Data are representative of three independent experiments.

3.2.14 Loss of Nrf2 leads to the dysregulated IL-10 production in DCs

Transcription of the anti-inflammatory cytokine IL-10 is regulated by CREB/ATF1 activity (Platzer et al., 1999). We therefore measured the levels of IL-10 secreted by the iDCs as readout of CREB/ATF1 transcriptional activity. As shown in **Figure 3.14*A***, $Nrf2^{-/-}$ iDCs produced higher levels of IL-10 in comparison to $Nrf2^{+/+}$ iDCs under basal conditions (40.1 pg/ml *versus* 25.7 pg/ml, $p < 0.05$). Furthermore, upon LPS stimulation, $Nrf2^{-/-}$ iDCs produced levels of IL-10, which was greater than that produced by LPS-stimulated $Nrf2^{+/+}$ iDCs (96.3 pg/ml *versus* 73.3 pg/ml, $p < 0.05$). Although basal production of IL-10 was not sensitive to p38MAPK inhibition, a significant reduction in LPS-induced IL-10 production ($Nrf2^{+/+}$ 73.3 pg/ml *versus* 31.3 pg/ml, $p < 0.05$; $Nrf2^{-/-}$ 96.3 pg/ml *versus* 41.2 pg/ml, $p < 0.05$) was observed in both $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs treated with SB203580 (**Figure 3.14*B***). This result suggests that LPS-stimulated but not basal IL-10 production in iDCs is dependent on p38MAPK-CREB activity.

Taken together, our findings suggest that the p38MAPK-CREB/ATF1 signalling axis contributes to the Nrf2-mediated regulation of DC immune function.

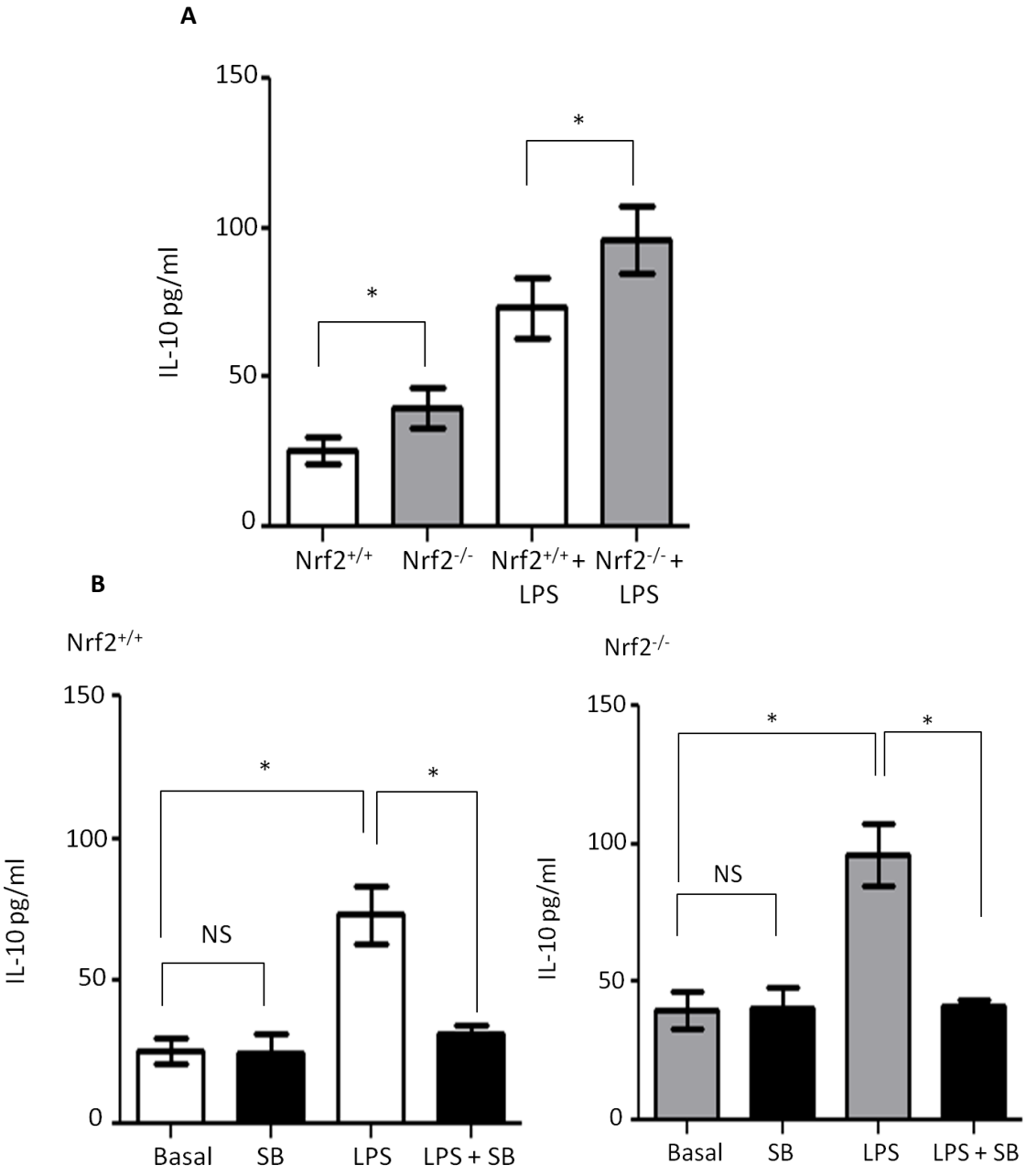


Figure 3.14. Loss of Nrf2 results in elevated IL-10 production by DCs. $Nrf2^{+/+}$ and $Nrf2^{-/-}$ DCs were incubated with or without (**A**) LPS (1 µg/ml) and/or (**B**) 20 µM SB203580 (SB) for 48 h. Levels of IL-10 in supernatants were measured by ELISA. Data are derived from two independent experiments and presented as average pg/ml ± S.D. Statistical significance was tested by one-way ANOVA (*, $p<0.05$; NS-Not Significant).

3.2.15 Increased DC co-stimulatory receptor expression due to the loss of Nrf2 is not dependent on PKA activity

In addition to p38MAPK and ERK1/2, CREB activity is also subject to regulation by PKA signalling in DCs (Kocieda et al., 2012; Mayr and Montminy, 2001). Therefore, we determined the contribution of PKA signalling to the aberrant DCs function associated with the absence of Nrf2 by utilising a pharmacological inhibitor of PKA (cAMPS-Rp) (Murray, 2008). **Figure 3.15*A*** and ***B***, showed that cAMPS-Rp treatment did not induce significant changes to co-stimulatory molecule expression in both $Nrf2^{+/+}$ (MHC class II 20.7 ± 2.4% *versus* 18.3 ± 2.6%, $p > 0.05$; CD86 17.4 ± 2.1% *versus* 19.4 ± 2.3%, $p > 0.05$) and $Nrf2^{-/-}$ (MHC class II 43.5 ± 1.9% *versus* 44.5 ± 1.8%, $p > 0.05$; CD86 45.3 ± 3.1% *versus* 47.7 ± 2.1%, $p > 0.05$) iDCs. This set of findings indicates that PKA activity is not required for DC co-stimulatory molecule expression.

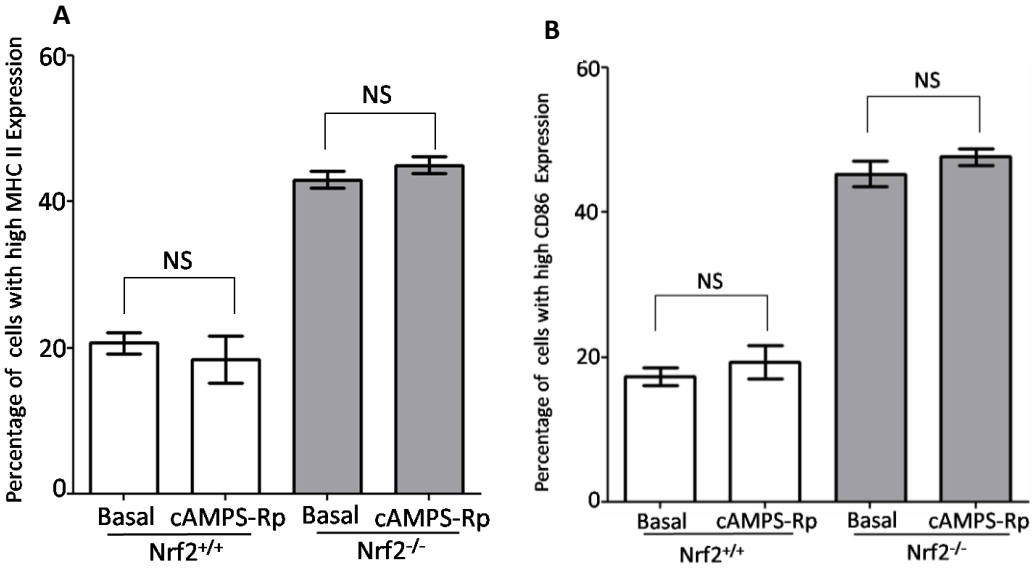
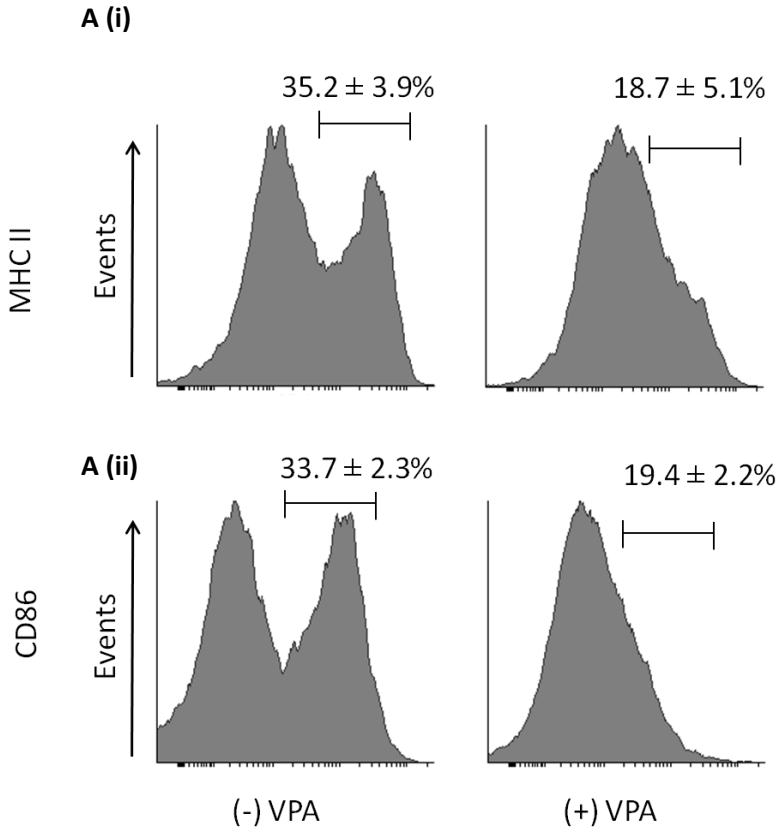


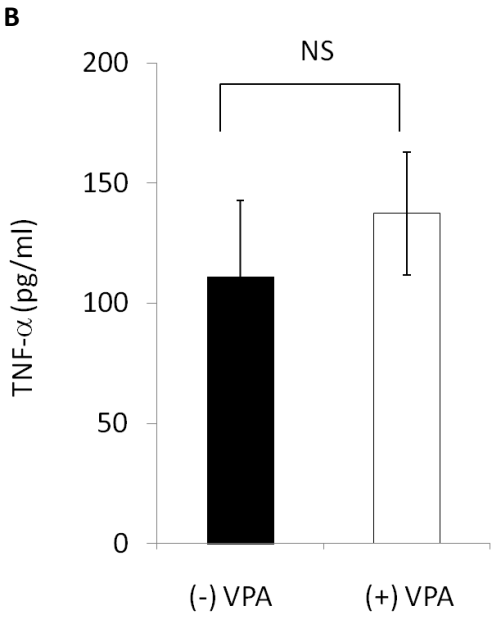
Figure 3.15. PKA is not required for Nrf2-dependent regulation of DC phenotype. $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs were treated with or without 15 µM of PKA inhibitor (cAMPS-Rp) for 48 h. MHC class II **(A)** and CD86 **(B)** expression was determined by flow cytometry and presented as percentage of cells expressing high MHC class II or CD86. Data derived from three independent experiments are presented as average percentage ± SD. Statistical significance was assessed using unpaired Student's *t* test. (NS-Not Significant).

3.2.16 Enhanced co-stimulatory receptor expression of Nrf2$^{-/-}$ iDCs is dependent on histone deacetylase activity

As the enhanced co-stimulatory molecule expression in Nrf2$^{-/-}$ iDCs was found to be regulated by transcription factors CREB and ATF1, we therefore explored whether other transcription regulators may also contribute. Histone deacetylases (HDACs) have multiple regulatory roles and originally identified as regulators of transcription (Woan et al., 2012). HDAC is involved in regulating DC differentiation, maturation, cytokine production, and immune function (Nencioni et al., 2007; Reddy et al., 2008). To investigate whether enhanced co-stimulatory molecule expression of Nrf2$^{-/-}$ iDCs was dependent on HDAC activity, valproic acid (VPA), an HDAC inhibitor (Minucci and Pelicci, 2006) was used. VPA at a concentration of 100 μg/ ml reduced iDC HDAC activity by around 50% (data not shown). As shown in **Figure 3.16*A***, VPA caused a reduction in MHC class II ***panel (i)*** and CD86 ***panel (ii)*** co-stimulatory molecule expression levels in the Nrf2$^{-/-}$ iDCs (MHC class II 35.2 ± 3.9% *versus* 18.7 ± 5.1%, $p < 0.05$; CD86 33.7 ± 2.3% *versus* 19.4 ± 2.2%, $p < 0.05$). In addition to altered co-stimulatory molecule expression, it has been previously shown that secretion of TNF-α is also dysregulated in Nrf2$^{-/-}$ iDCs (Rangasamy et al., 2010). We therefore were interested in whether TNF-α secretion is subject to modulation by HDAC inhibition in Nrf2$^{-/-}$ iDCs. Untreated Nrf2$^{-/-}$ iDCs secreted measurable quantities of TNF-α. However, inhibition of HDAC with VPA did not significantly alter TNF-α secretion (**Figure 3.16*B***). To examine whether the increased co-stimulatory molecule expression is accompanied by changes in HDAC activity, we measured HDAC activity in the nuclear lysates of Nrf2$^{+/+}$ and Nrf2$^{-/-}$ iDCs. Although there was a marginal increase in HDAC activity in the Nrf2$^{-/-}$ iDCs when compared with Nrf2$^{+/+}$ iDCs (0.80 ± 0.08 *versus* 0.74 ± 0.09 respectively), this was not statistically significant (**Figure 3.16*C***). Despite the absence of a marked increase in the HDAC

activity in the Nrf2$^{-/-}$ iDCs, our results suggest that the enhanced co-stimulatory molecule expression of Nrf2$^{-/-}$ iDCs is nevertheless dependent on HDAC activity.





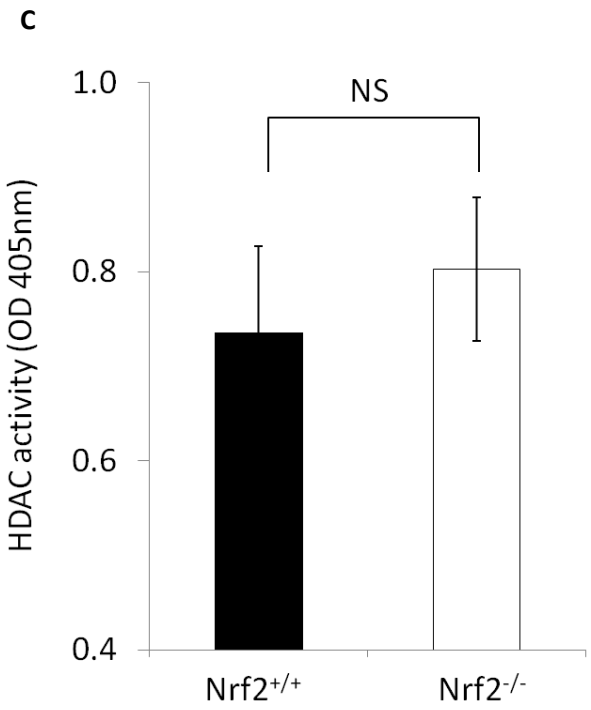


Figure 3.16. Histone deacetylase function is required for Nrf2-dependent regulation of DC phenotype. A, $Nrf2^{-/-}$ iDCs were treated with the HDAC inhibitor, valproic acid – 100 µg/ml (+VPA) for 72 hours, labelled with antibodies against MHC class II (**i**) and CD86 (**ii**) co-stimulatory receptors. The percentage of DCs expressing high levels of co-stimulatory receptors is indicated within the marker. Representative histograms are presented and average percentage ± SD is indicated above the markers. Data were derived from 4 independent experiments. **B,** $Nrf2^{-/-}$ iDCs were treated with VPA for 24 hours, supernatants collected and levels of TNF-α measured. Data are presented as average pg/ml ± SD and derived from 3 independent experiments. **C,** HDAC activity was measured in nuclear lysates from $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs. Data are presented as average absorbance at 405 nm ± SD and derived from 3 independent experiments

3.3 DISCUSSION

The work presented in this chapter investigated the role of the redox responsive transcription factor Nrf2 in DC immune function and intracellular signalling pathways. Dendritic cells are key antigen presenting cells that detect pathogens, process antigens and stimulate T cells to initiate and control a variety of immune outcomes.

DC immune functions can be potentially influenced by a variety of mechanisms including alterations of intracellular redox, induction of cytokine genes and modulation of intracellular signalling molecules. Nrf2 is central to redox homeostasis and understanding its role in DC biology requires the definition of critical molecular pathways that are subject to modulation by Nrf2 activity.

The lifespan of DCs can potentially influence immune responses by affecting the duration of lymphocytes stimulated by DCs (Chen et al., 2007; Izawa et al., 2007). After interacting with antigen-specific T cells, DCs may undergo accelerated clearance from the lymphoid organs. Failure to do so results in increase duration of T cells stimulation (Ingulli et al., 1997). Various DC subsets showed significant differences in their lifespan, however the molecular mechanisms that regulate such differences between DC subsets remain unclear (Chen et al., 2007). Increased susceptibility to infection was observed in conditions demonstrating reduced DC numbers (O'Shea et al., 2013) whilst on the other hand increased DC population was found in asthmatic and allergic respiratory diseases (Holt and Stumbles, 2000; Veres et al., 2013). This might contribute to Nrf2 related immune based diseases.
Nrf2 serves as a master regulator of stem cell integrity and longevity in adult tissues (Tsai et al., 2013). Additionally, another study has demonstrated a negative regulatory role for Nrf2 in intestinal stem cell proliferation where Nrf2 maintains homeostatic quiescence of intestinal stem cells in Drosophila. High ROS levels due

to faulty antioxidant mechanisms were linked to increased cell proliferation rates (Fiorani et al., 1995; Klaunig et al., 2010).

Our findings of increasing DC proliferation may highlight the role of Nrf2 in T cell tolerance and in preventing inappropriate adaptive immune response that could be responsible for developing autoimmune diseases.

Studies have shown that the co-stimulatory molecules MHC class II and CD86 are elevated in $Nrf2^{-/-}$ iDCs and our results support these findings (Rangasamy et al., 2010; Williams et al., 2008). We also found a marked increase in CD40 expression in the $Nrf2^{-/-}$ iDCs, which has not been documented previously. The difference in findings between the two studies could be due to some differences in DC culture methods and different strains of mice (C57BL6/SV129 *versus* CD1:ICR).

Immature DCs silence T cells either by deleting them or by expanding regulatory T cells. This minimizes the development of autoimmunity that may be triggered after infection or by self antigens captured from dying infected cells and harmless environmental proteins. However, when immature DCs express high levels of co-stimulatory molecules, this could lead to induction of autoimmunity and chronic immune-based diseases (Cools et al., 2007; Decker et al., 2006; Steinman and Nussenzweig, 2002).

Generation of functional cytotoxic T lymphocytes from naive CD8 T cells requires co-stimulatory and T cell receptor signals (Celluzzi and Falo, 1998; Chai et al., 1999; Kemball et al., 2006). CD8 T cells cannot be fully stimulated by immature DCs that normally express low amounts of co-stimulatory molecules (Bachmann et al., 1999b).

The potential ability of $Nrf2^{-/-}$ iDCs to stimulate enhanced CD8 T cell activation suggests that these DCs may not be tolerogenic. This suggests that Nrf2 is a potential immunoregulatory molecule involved in preventing inappropriate CD8 T cell immune activation by iDCs. It would be interesting to examine the effector

function of these CD8 T cells by looking at IFN-γ production to see whether CD8 T cells stimulated by Nrf2 deficient DCs had enhanced effector function. This would reaffirm that theses iDCs are not tolerogenic.

We have also shown in our lab that the increased DC co-stimulatory molecule expression was associated with impaired antigen acquisition capacity through endocytosis of dextran and phagocytosis of apoptotic and necrotic cells. This further reaffirms the enhanced maturation phenotype of the DCs as decreases in endo/phagocytosis capacity is associated with the maturation process (Aw Yeang et al., 2012).

In addition to the antigen surveying functions of immature DCs, iDCs also play a critical role in the T cell activation/tolerance balance in order to produce a closely tuned immune response with the clearance of infection or maintaining self-tolerance (Banchereau et al., 2000; Kurts et al., 1997a).
The implications of the enhanced co-stimulatory molecule expression by $Nrf2^{-/-}$ iDCs on antigen-specific T cell outcomes have not been explored. In this study, we have clearly demonstrated that $Nrf2^{-/-}$ iDCs presenting the antigenic peptides can induce substantial CD8 T cell proliferation in the absence of any further maturation or co-stimulatory signalling. Under normal conditions, presentation of self-peptides on MHC molecules to T cells by iDCs that express low levels of co-stimulatory molecules leads to the induction of T cell tolerance (Kurts et al., 1997a). Experiments conducted in our lab showed that NP34 (which is a partial agonist of NP68 and hence can mimic, to a degree the nature of a self-peptide) can induce F5 CD8 T cell activation by $Nrf2^{-/-}$ iDCs (Aw Yeang et al., 2012).

We and others have shown that in the absence of Nrf2, there is elevated ROS in DCs (**Figure 3.3**) (Aw Yeang et al., 2012; Rangasamy et al., 2010). Changes in ROS levels in DCs have been implicated in altering DC co-stimulatory molecule expression, maturation, and subsequent immune responses (Kantengwa et al., 2003; Kim et al.,

2008; Williams et al., 2008). Testing whether antioxidants could dampen ROS levels and reverse changes in function and co-stimulatory molecule expression of $Nrf2^{-/-}$ iDCs would clarify the role of ROS. Although there is evidence suggesting that increased ROS is associated with elevated co-stimulatory molecule expression (Kantengwa et al., 2003; Rutault et al., 1999), our results demonstrate that in the context of Nrf2 deficiency, ROS does not directly underlie these changes in immature DCs. A possible explanation for this is that increased co-stimulatory molecule expression in response to physiological stimuli is usually due to transient and not persistent elevation of ROS (Bergamo et al., 2008). The effect of chronic elevated basal levels of ROS in the intracellular environment of Nrf2-deficient DCs could be different from transient ROS elevations induced by chemical sensitizers. Sustained elevations of ROS levels as seen in $Nrf2^{-/-}$ iDCs are therefore likely to result in cellular adaptive changes that cannot be simply reversed by rebalancing ROS levels but require more complex cellular reprogramming (Boonstra and Post, 2004; Trachootham et al., 2009). Our findings suggest that Nrf2-mediated regulation of DC function is not solely dependent on its role in redox homeostasis. This is consistent with our previous results wherein lowering the levels of the redox-regulating molecule, GSH in $Nrf2^{+/+}$ iDCs does not recapitulate the altered phenotype and function of $Nrf2^{-/-}$ iDCs (Aw Yeang et al., 2012).

The transcription factor NF-κB is involved in DC development, survival, and maturation (Kim et al., 2010; Ouaaz et al., 2002; van de Laar et al., 2010). NF-κB is activated by a variety of stimuli such as LPS and oxidative stress (Rescigno et al., 1998). In unstimulated cells, NF-κB dimers remain inactive in the cytosol by the inhibitor of NF-κB proteins (IκBα) and upon activation, upstream IκB kinase (IKK) complexes are activated, which results in phosphorylation-induced proteosomal degradation of IκBα, leading to its ubiquitination. These events are essential upstream events required for NF-κB activation and this facilitates NF-κB translocation into the nucleus where it can transcribe genes involved in the immune

response (Basseres and Baldwin, 2006; Shih et al., 2011; van de Laar et al., 2010). ROS levels have been shown to both induce and repress NF-κB signalling depending on the cell type (Morgan and Liu, 2011; Zmijewski et al., 2007). Interestingly, our results revealed delayed kinetics of LPS-induced phosphorylation of p65 in $Nrf2^{-/-}$ iDCs. Elevated ROS levels in $Nrf2^{-/-}$ iDCs could induce modifications in the cysteine residues in upstream kinases, namely IKK β, resulting in its inactivation and inability to phosphorylate phospho-65 (Reynaert et al., 2006). However, we did not find any difference in basal NF-κB activation and it appears that changes to upstream elements of the NF-κB pathway in $Nrf2^{-/-}$ iDCs do not translate to alterations in nuclear NF-κB levels. This is consistent with previous studies in Nrf2-deficient mice whereby no differences in basal NF-κB activation in lung lysates were observed between $Nrf2^{-/-}$ and wild type mice (Rangasamy et al., 2005; Thimmulappa et al., 2006). Furthermore, IL-10 was found to block NF-κB activity through the suppression of IKK activity and inhibition of NF-κB DNA binding activity (Schottelius et al., 1999). Therefore, this might be the cause for the delayed degradation of IκBα in Nrf2-deficient DCs as they produced more IL-10. Additionally, inhibiting NF-κB could not reverse the enhanced co-stimulatory molecule expression and CD8 T cell stimulation capacity in the Nrf2-deficient DCs. This further reaffirms that NF-κB pathway does not contribute to the altered $Nrf2^{-/-}$ iDC immune function.

Increased intracellular ROS and other extracellular stimuli such as LPS also induce activation of MAPKs, resulting in increased DC co-stimulatory molecule expression, cytokine production, and capacity to stimulate T cells in secondary lymphoid organs (Nakahara et al., 2006). Previous inhibitor studies have implicated ERKs and p38MAPK in CD86 and MHC class II expression in human DCs under conditions where changes in ROS have been induced by chemical sensitizers (Aiba et al., 2003). Our results showed that, by using pharmacological inhibitors, ERK and JNK were not responsible for the altered phenotype and function in the Nrf2-deficient DCs despite the enhanced basal phosphorylation status of both MAPKs.

Our observations suggest that the p38MAPK but not ERK1/2 or JNK is the main MAPK that is involved in Nrf2-mediated regulation of immature DC function. Consistent with our findings, enhanced Nrf2 activity through the use of sulforaphane has been shown to suppress p38MAPK pathway in endothelial cells (Zakkar et al., 2009). The inhibition of p38MAPK causes a significant but incomplete reversal of DC function in Nrf2-deficient DCs, indicating that other pathways or factors are also involved. Indeed, histone deacetylases have also been implicated in the Nrf2-associated changes in phenotype (Aw Yeang et al., 2012). Increased and/or prolonged stimulation of MAPKs signalling pathways is linked to autoimmune diseases such as rheumatoid arthritis and psoriasis, and inhibitors of these pathways represent new therapeutic strategies (Cohen, 2009; Huang et al., 2012). It will be interesting to examine the levels of Nrf2 in these conditions.

The downstream target of p38MAPK, CREB, has been associated with the regulation of key DC immune functions, including the expression of co-stimulatory molecules (Ardeshna et al., 2000). Our findings of constitutive basal hyperphosphorylation of CREB and ATF1 in $Nrf2^{-/-}$ iDCs highlight the requirement of Nrf2 in maintaining the integrity of the p38MAPK-CREB/ATF1 pathway in iDCs. Using pharmacological inhibitors, we confirmed that CREB and ATF1 hyperphosphorylation was selectively mediated by p38MAPK and not ERK1/2. The other MAPK, JNK was not involved in CREB/ATF1 phosphorylation (Vaishnav et al., 2003) and hence was not further examined in this study. In keeping with the lack of involvement of ROS in altered co-stimulatory molecule expression, reducing ROS did not alter CREB/ATF1 phosphorylation levels. These observations confirm the selective involvement of p38MAPK-CREB/ATF1 axis in Nrf2-mediated regulation of DC function.

We also discounted another major activator of CREB, PKA, in mediating the changes in co-stimulatory molecule expression that accompanies loss of Nrf2. The cAMP-dependent protein kinase signalling transduction cascade is highly conserved among a wide variety of organisms. The major targets of PKA are the transcription

factors of the cAMP response element binding (CREB) family and this represents cross-talk between intracellular signalling pathways (Naviglio et al., 2009). In DCs, cAMP/PKA pathway is involved in promoting DC maturation via upregulation of co-stimulatory molecules and increasing IL-10 with inhibiting IL-12 and TNF-α production (Schillace et al., 2009). Therefore, it would be important to identify its contribution to increase co-stimulatory molecule expression via aberrant CREB/ATF1 phosphorylation. Our results using selective inhibitors suggest that cAMP-PKA had no effect on the increased co-stimulatory molecules seen in $Nrf2^{-/-}$ iDCs.

The phosphorylation state of p38MAPK-CREB/ATF1 axis can be regulated by protein phosphatases (Chi et al., 2006; Johannessen et al., 2004). Key phosphatases that act on p38MAPK-CREB/ATF1 axis and regulate their phosphorylation are MKP-1, PP1 (Hagiwara et al., 1992) and PP2A (Shanware et al., 2010). It is possible that changes in MKP-1, PP1, and PP2A activity could also contribute to the increased phosphorylation of p38MAPK-CREB/ATF1 axis in $Nrf2^{-/-}$ iDCs. It will be interesting to measure MKP-1, PP1 and PP2A activity in these DCs to test this possibility.

$Nrf2^{-/-}$iDCs produce higher levels of IL-10 in comparison to $Nrf2^{+/+}$ iDCs. IL-10 has a crucial effect as a feedback regulator of diverse immune responses for both Th1 as well as Th2 cell responses and this evidence links overproduction of IL-10 to many pathological conditions like systemic lupus erythematosus (SLE) (Liu et al., 2011). It is already established that aged Nrf2 deficient female mice develop SLE like disease and this could be in part due to high circulating levels of IL-10 (Lee et al., 2009).

There is evidence that stimuli-induced IL-10 production requires CREB/ATF1 activation (Platzer et al., 1999). Surprisingly, we found that the basal IL-10 production could not be reduced by inhibition of the p38MAPK-CREB/ATF1 pathway. However, LPS-induced IL-10 secretion was sensitive to inhibition of this pathway. This suggests that there are differential requirements for p38MAPK-

CREB/ATF1 in the transcriptional regulation of IL-10 under basal versus stimulated conditions. It is pertinent to note that although the level of CREB/ATF1 phosphorylation in $Nrf2^{-/-}$ iDCs under basal conditions was comparable with that in LPS-stimulated iDCs, the amount of IL-10 secreted basally was only half of that in LPS-stimulated iDCs. This indicates that CREB/ATF1 phosphorylation alone was not sufficient for IL-10 synthesis. A direct functional consequence of increased CREB/ATF1 phosphorylation in $Nrf2^{-/-}$ iDCs include increased expression of certain target genes like levels of Bcl-2 (Sakamoto and Frank, 2009). This explains the increased expression of Bcl-2 observed in this study. Transactivation by CREB/ATF1 requires its association with cofactors such as CREB binding protein (CBP) (Cardinaux et al., 2000). There are data demonstrating that availability of cellular CBP is limited and that CBP is also utilized by other transcription factors, including Nrf2 and NF-κB (Alvarez et al., 2009). It is therefore suggested that Nrf2 and NF-κB (in addition to CREB) compete for the available CBP (Gerritsen et al., 1997; Katoh et al., 2001; Vo and Goodman, 2001). In the absence of Nrf2, more CBP is available for use by NF-κB and CREB. This could partly explain the increased levels of a number of genes transcribed by these transcription factors in $Nrf2^{-/-}$ iDCs (Jin et al., 2008; Thimmulappa et al., 2006). This duration of a CREB-dependent transcriptional event may be determined, at least in part, by the nature of the dimer bound to the target promoter cAMP responsive element (CRE) (Shaywitz and Greenberg, 1999).

As a transcription factor, CREB, is a challenging target for inhibition by small molecules, however various small molecules have been studied as CREB inhibitors either to inhibit CREB phosphorylation, CREB-CBP interaction, or CREB-DNA binding. These approaches are associated with off-target effects probably because CREB is a focal point of many different signalling pathways and there is a risk of DNA damage (Xiao et al., 2010).

Our data, in conjunction with other studies, suggest that Nrf2 activity can affect cell signalling both at the level of p38MAPK-CREB/ATF1 and at the level of transcriptional cofactors.

HDAC has been associated with modulating DC differentiation, co-stimulatory molecule expression, cytokine production, and immunogenicity (Nencioni et al., 2007). Our finding that the Nrf2-deficient co-stimulatory molecule expression can be reversed by HDAC inhibition places HDACs as potential targets of Nrf2 function. Although the HDAC activity in $Nrf2^{-/-}$ iDCs tended to be higher than in the wild type DCs, this difference was not statistically significant. However, it is possible that such small differences could lead to significant biological outcomes. In particular, a marginally higher HDAC function can lead to cumulative effects over time and may drive epigenetic changes, thus altering co-stimulatory molecule expression on DCs. Interestingly, dysregulation of HDAC functions has been associated with diseases such as cancer, chronic obstructive pulmonary disease, and asthma in which the pathology is associated with redox disequilibrium, implicating the link between redox status and HDAC (Chung and Marwick, 2010; Minucci and Pelicci, 2006). Further investigation into the molecular pathways that lead from Nrf2 activity to ROS through to HDAC is merited. The availability of pharmaceuticals that target HDACs for therapeutic indications opens up the possibility of using such therapeutic strategies to modulate DC functions (Faraco et al., 2011; Reddy et al., 2008; Vojinovic and Damjanov, 2011) in diseases that are associated with altered Nrf2 function.

In summary, our findings indicate the importance of the transcription factor Nrf2 in maintaining the integrity of key intracellular signalling pathways important for normal DC function and implicates p38MAPK-CREB/ATF1 axis as a key signalling pathway that is regulated by Nrf2 in DCs. The molecules within this pathway could represent targets for pharmacological intervention in disease states that arise from dysregulated Nrf2 function.

For these reasons, shaping of the adaptive immune responses by manipulating DC biology has been gaining huge research interests for translational therapeutic purposes.

CHAPTER FOUR

REGULATION OF DC PHENOTYPE, FUNCTION, AND SIGNALLING PATHWAYS BY HEME OXYGENASE-1

4.1 INTRODUCTION

Heme oxygenases (HOs) are intracellular rate-limiting enzymes which catalyse the degradation of cellular heme into biliverdin (BV), carbon monoxide (CO), and free iron. Three distinct HO enzymes have been identified: HO-1, HO-2, and HO-3 (Siow et al., 1999). HO-1 is an inducible stress-response protein whose expression is induced mainly by heme in addition to a variety of oxidative stimuli including heavy metals, ultraviolet irradiation, hyperthermia, hydrogen peroxide, inflammatory cytokines, and nitric oxide (Naito et al., 2011; Ryter and Choi, 2009; Takahashi et al., 2009). The role of heme is a double-edged sword; on one hand it is required for vital biological functions and enzymatic reaction of many heme-containing apoproteins such as hemoglobin, myoglobin and cytochrome-c oxidase (Padmanaban et al., 1989; Wijayanti et al., 2004). While on the other hand free heme catalyzes the formation of reactive oxygen species (ROS) leading to oxidative stress-induced cellular and tissue damage. Therefore, intracellular generation of heme is required to be tightly regulated (Ponka, 1999; Sassa, 2004). Cells use multiple signalling pathways and transcription factors to fine-tune intracellular levels of heme. Among these factors, the Nrf2 transcription factor is the most important for transcription of HO-1 and the repressor factor Bach1 acts as a negative regulator (Alam and Cook, 2007).

HO-1 has a cytoprotective effect against oxidative insults and inflammation. The induction of HO-1 expression through gene transfer or pharmacological activations has had therapeutic efficacy in a variety of conditions or disorders involving the immune system like animal models of organ transplantation and inflammatory disorders (Ryter et al., 2006).

Dendritic cells (DCs) are potent antigen presenting cells that play a major role in the initiation and regulation of the immune response (Steinman, 2003). Depending on their maturation state, DCs can modulate immune functions via changes in its

migratory capacity, co-stimulatory molecule expression, and antigen presentation abilities (Banchereau and Steinman, 1998). Dendritic cell maturation and function is influenced by changes in cellular levels of reactive oxygen species (ROS) (Rutault et al., 1999). Alterations in intracellular ROS can also impact on the activity of the p38MAPK pathway (Matos et al., 2005). Heme is a product of catabolism of heme-containing metalloproteins that has the potential to induce the generation of ROS (Jeney et al., 2002).

HO-1 has been shown to inhibit lipopolysaccharide (LPS)-induced DC maturation and expression of pro-inflammatory cytokines (Chauveau et al., 2005; Cheng et al., 2010). Furthermore, lack of HO-1 results in profound immune dysregulation and induces DC maturation while induction of HO-1 expression renders DCs to be in an immature state (Chauveau et al., 2005; Cheng et al., 2010; Park et al., 2010). Given what is known about the role of HO-1 in DCs, it is unclear whether HO-1 utilises the p38MAPK pathway to mediate regulation of DC maturation and subsequent immune functions. Furthermore, it is not known whether the primary effect of HO-1 in regulating DC maturation is by preventing elevation of intracellular ROS levels. Finally, it is unknown whether the HO-1 substrate, heme is directly involved in regulating DC maturation and function through activation of the p38MAPK-CREB/ATF1 pathway. The objective for this segment of research was to address the above mentioned gaps in knowledge.

4.2 RESULTS

4.2.1 Loss of Nrf2 results in decreased expression of HO-1

We previously noted that the loss of Nrf2 results in the dysregulation of DC immune functions (Aw Yeang et al., 2012). We were interested in indentifying downstream products that were modulated by Nrf2 in these DCs. Nrf2 regulates a group of

stress-inducible protein genes including HO-1 genes (Alam et al., 1999). To investigate the capacity of DCs to express HO-1 in the absence of Nrf2, we assessed the basal level of HO-1 in iDCs from wild type and $Nrf2^{-/-}$ mice by Western blotting. Loss of Nrf2 is found to reduce the expression HO-1 in comparison to its wild type counterpart as shown in **Figure 4.1**.

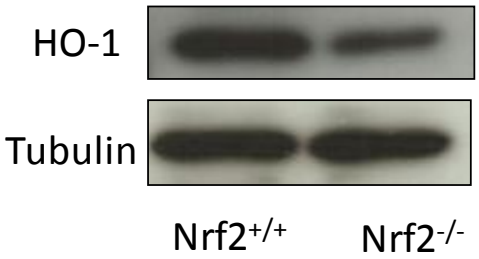


Figure 4.1. Loss of Nrf2 results in reduced expression of HO-1. Whole cell lysates from $Nrf2^{+/+}$ and $Nrf2^{-/-}$ iDCs were subjected to SDS-PAGE. Expression of HO-1 was assessed by Western blotting. Tubulin was used as loading control. Data are representative of three independent experiments.

4.2.2 HO-1 expression decreases during DC maturation

Human and rat DC maturation was associated with decreased expression of HO-1 (Chauveau et al., 2005). Given the inducible and dual nature of HO-1 we next asked the question if HO-1 can be self-modulated during normal physiological processes within bone marrow-derived DCs. One basic physiologic action of DCs is the process of maturation and this process has been shown to be metabolically taxing to the cells (Banchereau and Steinman, 1998; Rangasamy et al., 2010) which may invoke the transcription of HO-1 (via Nrf2). Western immunoblotting of bone marrow-DCs shows that murine iDCs expressed HO-1 and this expression was decreased when DCs matured by inflammatory stimuli (LPS) (**Figure 4.2**).

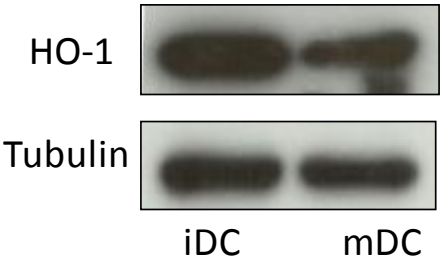
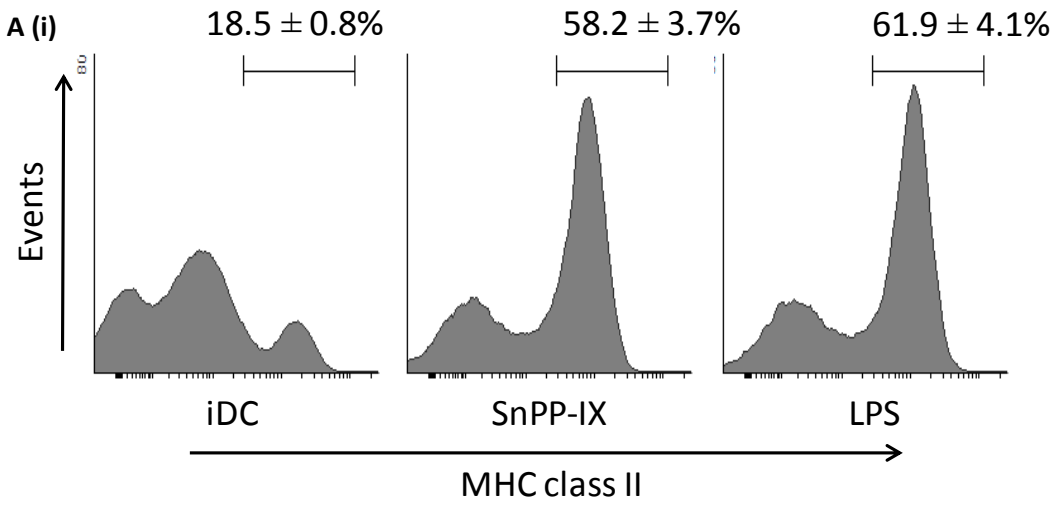


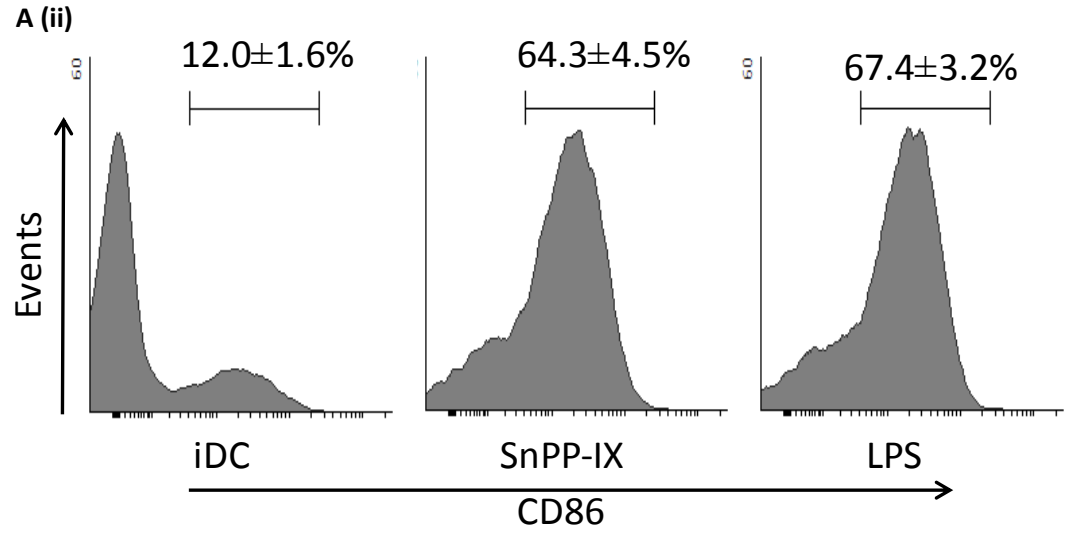
Figure 4.2. DC maturation is associated with reduction in HO-1 expression. Whole cell lysates were generated from untreated (iDC) or treated with LPS 1 μg/ml (mDC) for 18 h and subjected to SDS-PAGE. Expression of HO-1 was assessed by Western blotting. Tubulin was used as loading control. Data are representative of three independent experiments.

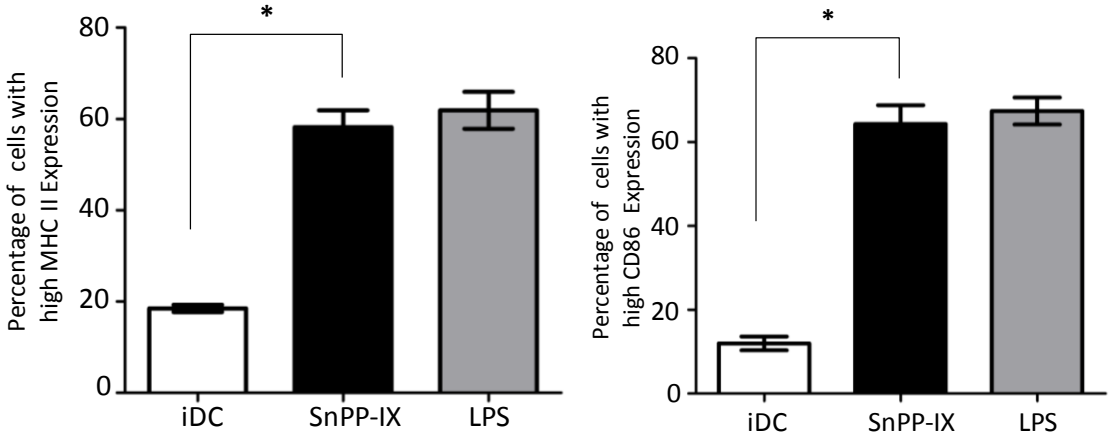
4.2.3 Inhibition of HO-1 activity alters immature DC phenotype and function

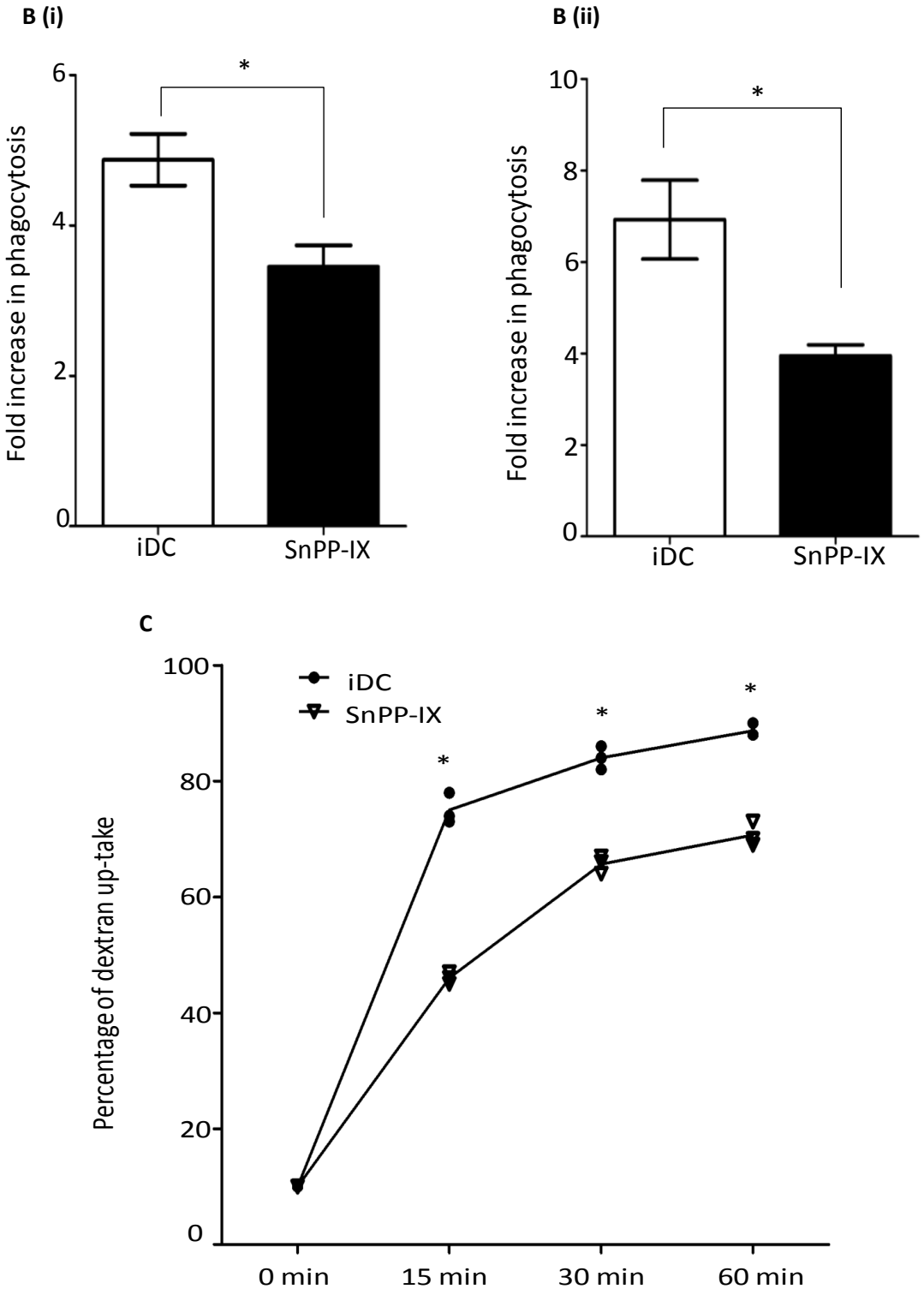
To investigate the effect of HO-1 activity on DC immune function, we treated DCs with tin protoporphryin-IX dichloride (SnPP-IX), a HO-1 inhibitor (Kappas et al., 1988) and then evaluated DC phenotype and function. **Figure 4.3*A*** showed that iDCs treated with (SnPP-IX) showed high levels of cell surface co-stimulatory molecule expression when compared to untreated iDCs (MHC class II, 58.2 ± 3.7% *versus* 18.5 ± 0.8%, $p<0.05$, ***panel i***; CD86, 64.3 ± 4.5% *versus* 12.0 ± 1.6%, $p<0.05$, ***panel ii***). Furthermore, MHC class II and CD86 levels on SnPP-IX-treated iDCs were comparable to LPS-treated iDCs (MHC class II 61.9 ± 4.1% and CD86 67.4 ± 3.2%). These findings suggest that inhibition of HO-1 activity results in the DC maturation when induced by LPS. Following maturation, DCs have reduced phagocytic and endocytic capabilities (Banchereau et al., 2000; Rescigno et al., 1999). As HO-1 inhibition resulted in DC maturation, we examined the antigen acquisition capacity of SnPP-IX-treated iDCs. Our results revealed that SnPP-IX-treated iDCs had a reduced capacity to phagocytose both necrotic (**Figure 4.3*B panel i***, 4.9 ± 0.7 *versus* 3.4 ± 0.6 -fold increase over baseline, $p < 0.05$) and apoptotic cells (**Figure 4.3*B***

panel ii, 6.9 ± 1.7 *versus* 4.0 ± 0.5 -fold increase over baseline, $p < 0.05$) compared to their untreated control. Results also demonstrated that SnPP-IX-treated iDCs had a diminished capacity to endocytose dextran in comparison to their untreated controls (**Figure 4.3*C***, 46.0 ± 1.0% *versus* 75.0 ± 2.6% at 15 min, 65.7 ± 1.5% *versus* 84.0 ± 2.0% at 30 min, and 70.7 ± 2.1% *versus* 88.7 ± 1.2% at 60 min, $p < 0.05$). Low levels of co-stimulatory molecule expression in iDCs render them unable to stimulate a fully competent antigen-specific CD8 T cell response (Bachmann et al., 1999b). However, up-regulation of these surface molecules in mature DCs enhances their ability to induce T cell activation (Lanzavecchia and Sallusto, 2001). As SnPP-IX-treated iDCs exhibit a mature phenotype, we expected that this would be associated with an enhanced capacity to induce DC-mediated antigen-specific CD8 T cell activation. To test this, we utilised a TCR transgenic mouse model, wherein the CD8 T cells exclusively express the F5 T cell receptor (F5 TCR) that specifically recognise the MHC class I ($H2\text{-}D^b$) restricted antigenic peptide, NP68, when presented by DCs (Mamalaki et al., 1992). Functional consequences of altered DC co-stimulatory receptor expression were assessed by the ability of NP68 bearing DCs to stimulate antigen-specific F5 CD8 T cell proliferation. We observed that SnPP-IX-treated iDCs elicited enhanced DC-mediated antigen-specific F5 CD8 T cell proliferation in relation to the untreated control at all NP68 concentrations as shown in **Figure 4.3*D*** (2.5 fold at 1 and 10 nM to 1.4 fold at 100 nM, $p < 0.05$). Taken together, these findings indicate that inhibition of HO-1 activity affects DC phenotypic maturation, antigen acquisition ability, and antigen-specific CD8 T cell stimulatory capacity.


A (i)
18.5 ± 0.8%
58.2 ± 3.7%
61.9 ± 4.1%
Events
iDC
SnPP-IX
LPS
MHC class II


A (ii)
12.0±1.6%
64.3±4.5%
67.4±3.2%
Events
iDC
SnPP-IX
LPS
CD86


*
Percentage of cells with high MHC II Expression
80
60
40
20
0
iDC
SnPP-IX
LPS
*
Percentage of cells with high CD86 Expression
80
60
40
20
0
iDC
SnPP-IX
LPS


B (i)
Fold increase in phagocytosis
6
4
2
0
*
iDC
SnPP-IX
B (ii)
Fold increase in phagocytosis
10
8
6
4
2
0
*
iDC
SnPP-IX
C
Percentage of dextran up-take
100
80
60
40
20
0
iDC
SnPP-IX
*
*
*
0 min
15 min
30 min
60 min

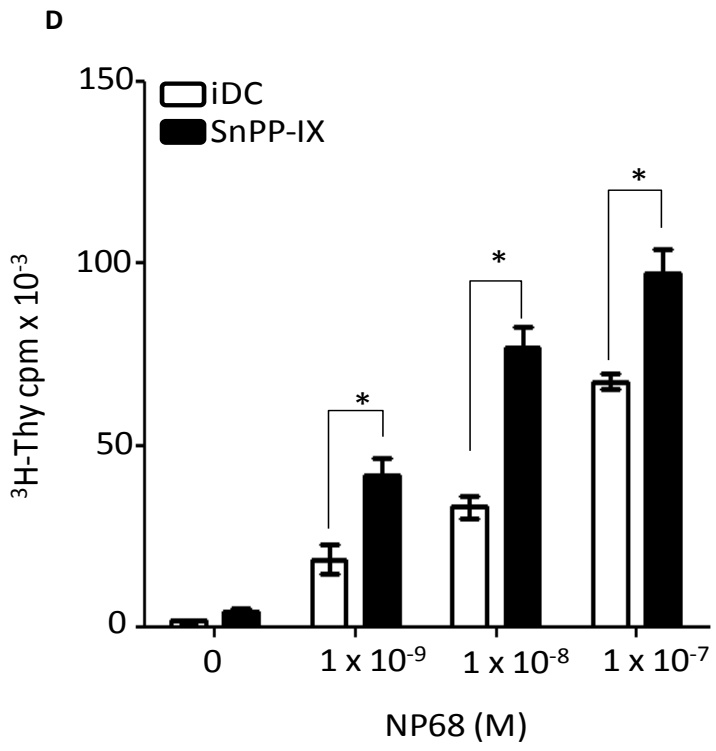


Figure 4.3. Inhibiting HO-1 activity alters dendritic cell phenotype and function. A, Immature DCs untreated or treated with HO-1 inhibitor (SnPP-IX, 5 µM) or (1 µg/ml) LPS for 14 h. Cells were labelled with fluorescent conjugated antibodies against MHC class II and CD86 co-stimulatory molecules. Co-stimulatory molecule expression was determined by flow cytometry. The percentages of iDCs expressing high levels of MHC class II (**i**) and CD86 (**ii**) are indicated above the marker. Representative histograms are presented with average percentage ± SD. (*, $p < 0.05$). Data are derived from three independent experiments. **B,** Immature DCs untreated or treated with SnPP-IX (5 µM) for 14 h were co-cultured with CFSE-labelled necrotic Jurkat cells (**i**) or apoptotic thymocytes (**ii**) at 37 °C for 2 h. DC phagocytic capacity was measured by flow cytometry as an increase in CFSE levels when compared with corresponding 4°C baseline control samples. Data derived from four independent experiments are presented as average-fold changes ± S.E.M. Statistical significance was tested by Mann-Whitney U test (*, $p < 0.05$). **C,** Endocytic capacity was measured by incubating iDCs untreated or treated with SnPP-IX (5 µM) for 14 h with Dextran^{FITC} for the indicated time points at 37°C. Dextran^{FITC} uptake by iDCs was assessed by flow cytometry. Data derived from three independent experiments are presented as average percentage of uptake ± S.D. Statistical significance was tested by unpaired Student's t test (*, $p < 0.05$). **D,** Immature DCs untreated or treated with SnPP-IX (5 µM) for 14 h were pulsed with increasing concentrations of NP68 antigenic peptide, and co-cultured with F5 CD8 T cells for 72 h. [^{3}H]-Thymidine (^{3}H-Thy) was added for the last 16 h. Proliferation of T

cells was determined by scintillation counting of incorporated ^{3}H-Thy. Data are presented as average scintillation counts ± S.D. Statistical significance was assessed using one-way ANOVA. Data are representative of three independent experiments (*, $p < 0.05$).

4.2.4 HO-1 inhibition in DCs results in elevated intracellular ROS levels

Dendritic cell maturation and function is influenced by intracellular ROS levels (Kantengwa et al., 2003). Heme, substrate for HO-1 activity, can enhance ROS generation (Figueiredo et al., 2007). In order to test whether HO-1 activity is required for regulation of ROS levels in DCs, we treated iDCs with SnPP-IX and measured the ROS levels by flow cytometry. We found that HO-1 inhibition for 14 hours resulted in a significant increase in intracellular ROS levels in iDCs as shown in **Figure 4.4*A*** (mean fluorescence intensity (MFI) 449.6 ± 16.2 *versus* 178.2 ± 2.9, $p < 0.05$). However, this increment in ROS levels was less than that observed in the LPS-treated DCs (MFI 615.2 ± 24.2). Intracellular ROS can be lowered by the ROS scavengers vitamins C and E (Al-Huseini et al., 2013; Cachia et al., 1998; Tan et al., 2005) and indeed co-treatment with vitamins C and E resulted in significant reduction in ROS levels in SnPP-IX-treated iDCs (**Figure 4.4*B***, MFI 254.2 ± 2.9 *versus* 449.6 ± 16.2, $p < 0.05$). These findings implicate HO-1 activity in preventing elevation of intracellular ROS levels in iDCs.

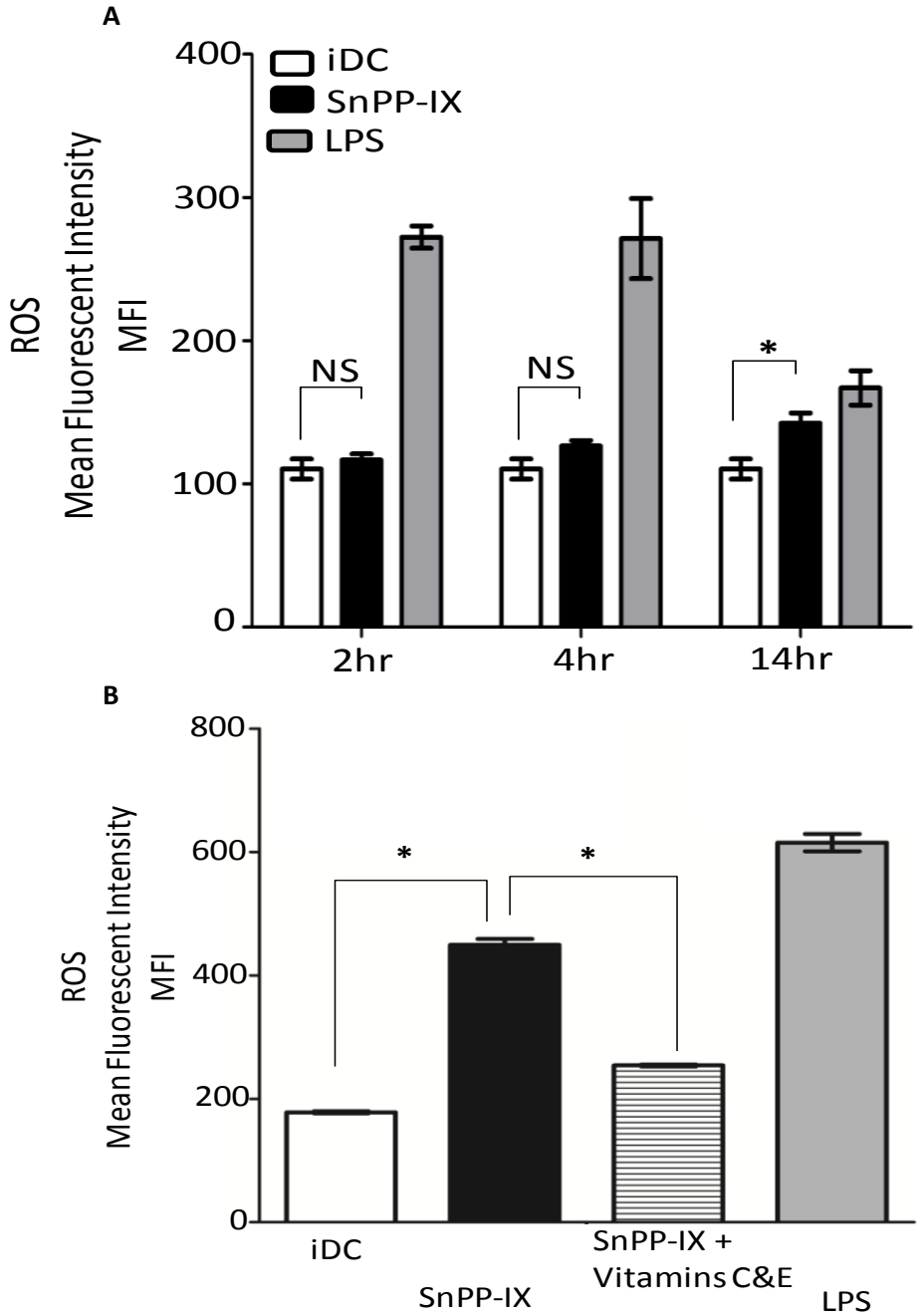


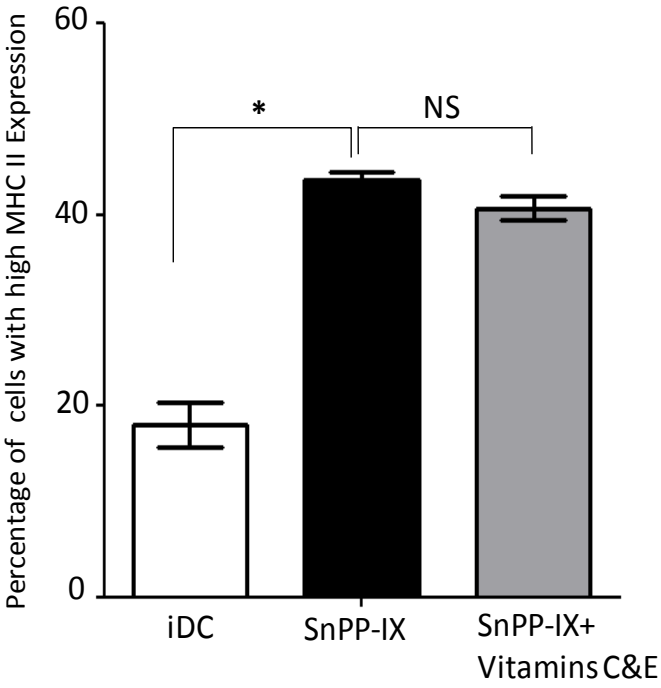
Figure 4.4. Inhibiting HO-1 activity increases ROS levels in DCs that can be restored by vitamin treatment. A, Immature DCs untreated or treated with tin protoporphyrin IX dichloride (SnPP-IX, 5 µM), or LPS (1 µg/ml) for indicated time points. Cells were incubated with the ROS indicator dihydroethidium and analysed by flow cytometry. Data are presented as average mean fluorescence intensity ± S.D and derived from three independent experiments (*, $p < 0.05$; NS, not significant). **B**, Immature DCs untreated or treated with SnPP-IX (5 µM) for 14 h or with a combination of vitamins C (1 mM) and E (100 µM) for 48 h and SnPP-IX (5 µM) for 14 h, or with LPS (1 µg/ml) for 2 h. DCs were incubated with the fluorescent ROS indicator, dihydroethidium and analysed by flow cytometry. Data derived from

three independent experiments are presented as average mean fluorescence intensity ± S.D (*, $p < 0.05$).

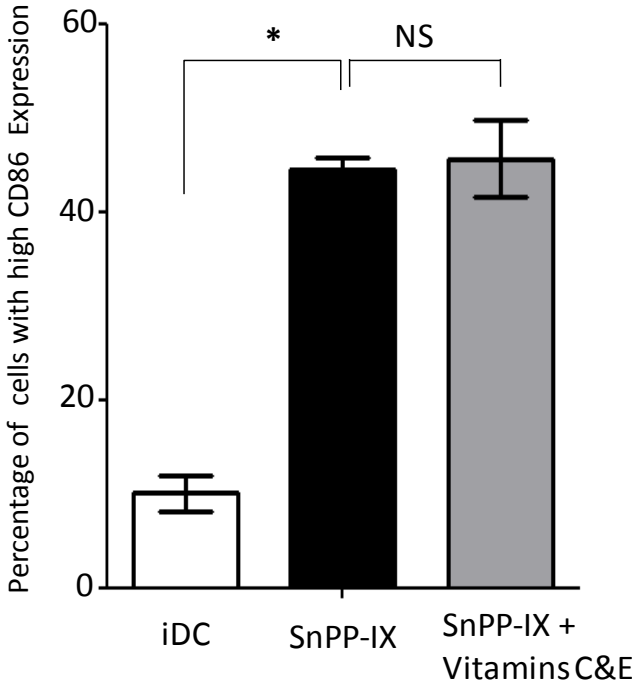
4.2.5 Altered immature DC function by HO-1 inhibition is not dependent on elevated ROS

To test whether DC maturation induced by HO-1 inhibition was a result of increased intracellular ROS levels, we treated HO-1 inhibited iDCs with vitamins C and E and examined MHC class II and CD86 expression. Our results revealed that there were no significant differences between SnPP-IX treatment alone or in combination with vitamins C and E in iDC expression of MHC class II (**Figure 4.5*A panel i***, 43.6 ± 0.8 *versus* 40.7 ± 1.3, $p > 0.05$) and CD86 (**Figure 4.5*A panel ii***, 44.5 ± 1.4 *versus* 45.7 ± 4.1, $p > 0.05$). Similarly, there were no differences observed in iDC-mediated antigen-specific F5 CD8 T cell proliferation upon SnPP-IX treatment alone or in combination with vitamins C and E (**Figure 4.5*B***). These observations demonstrate that the altered iDC phenotype and function induced by HO-1 inhibition is not a result of elevated ROS in these iDCs.

A (i)


Percentage of cells with high MHC II Expression
60
40
20
0
*
NS
iDC
SnPP-IX
SnPP-IX+
Vitamins C&E

A (ii)


Percentage of cells with high CD86 Expression
60
40
20
0
*
NS
iDC
SnPP-IX
SnPP-IX +
Vitamins C&E

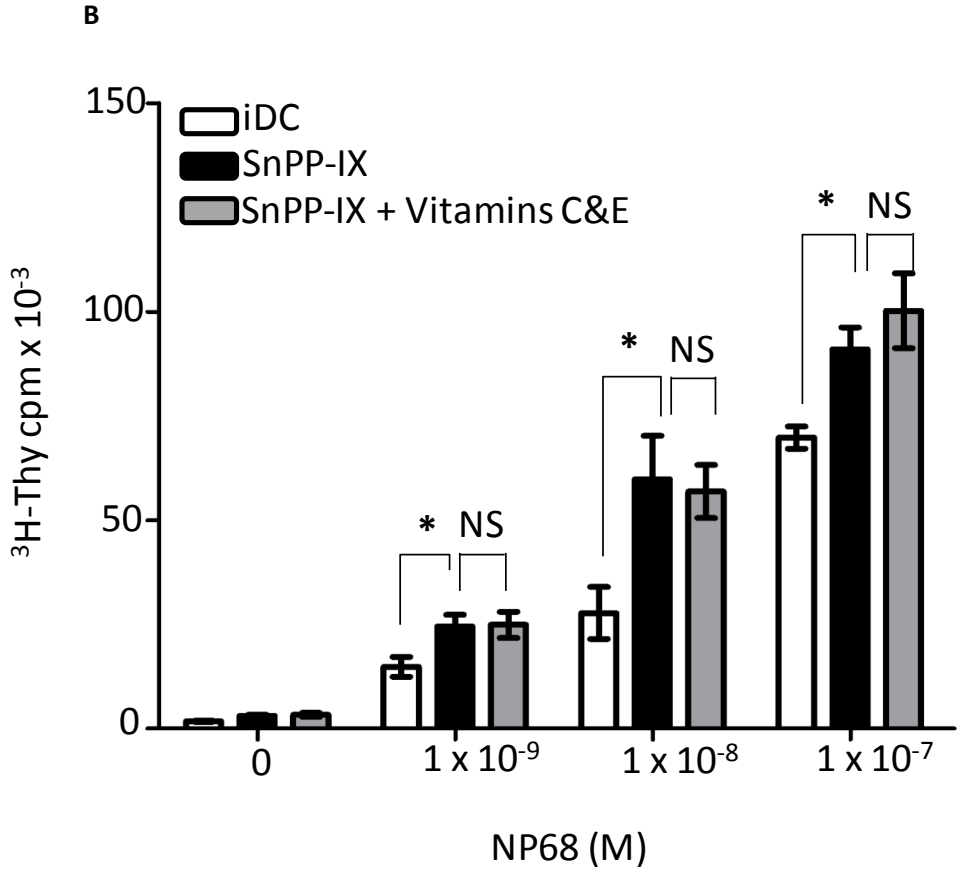
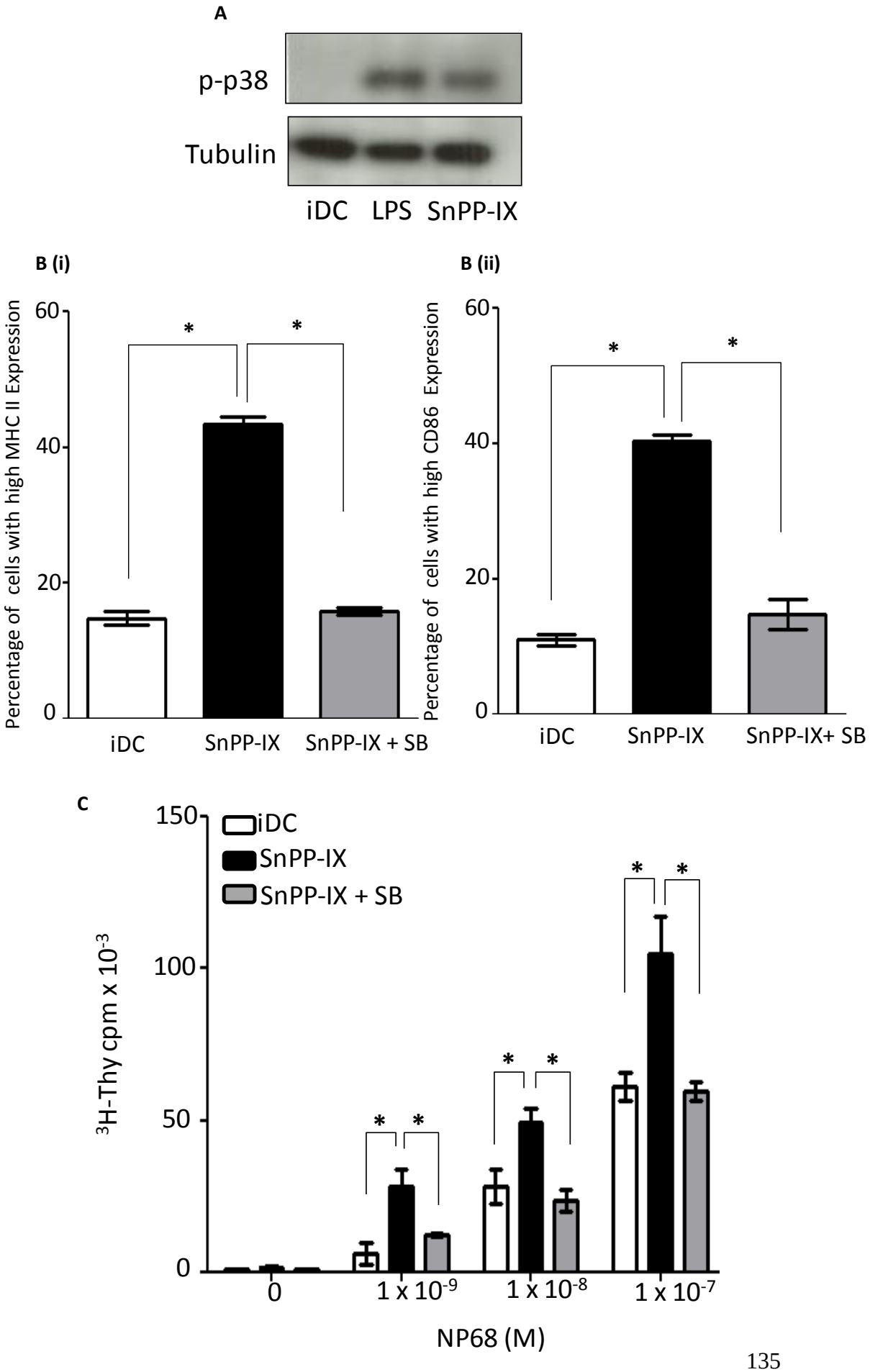


Figure 4.5. Altered immature DC function by HO-1 inhibition is not dependent on elevated ROS. A, Immature DCs untreated or treated with SnPP-IX (5 µM) for 14 h alone, or with a combination of vitamins C (1 mM) and E (100 µM) for 48 h plus SnPP-IX (5 µM) for 14 h. MHC class II (**i**) and CD86 (**ii**) expression were determined by flow cytometry and presented as percentage of cells expressing high MHC class II or CD86. Data derived from three independent experiments are presented as average percentage ±S.D. (*, $p < 0.05$; NS, not significant). **B**, Immature DCs untreated or treated with SnPP-IX (5 µM) for 14 h alone, or treated with vitamins C (1 mM) and E (100 µM) for 48 h along with SnPP-IX (5 µM) for the last 14 h. Cells were pulsed with increasing concentrations of NP68 antigenic peptide, and co-cultured with F5 CD8 T cells for 72 h. [^{3}H]-Thymidine (^{3}H-Thy) was added for the last 16 h. Proliferation of T cells was determined by scintillation counting of incorporated ^{3}H-Thy. Data are presented as average scintillation counts ± S.D. Statistical significance was assessed using one-way ANOVA. Data are representative of three independent experiments (*, $p < 0.05$; NS, not significant).

4.2.6 HO-1 regulates DC phenotype and function through the p38MAPK-CREB/ATF1 pathway

Activation of the p38MAPK-CREB/ATF1 signalling pathway has been shown to be involved in DC maturation (Arrighi et al., 2001). Activation of this pathway is accompanied by an increase in the serine phosphorylation status of p38MAPK (Ardeshna et al., 2000; Shanware et al., 2010). In order to investigate whether p38MAPK is involved in the HO-1 mediated regulation of DC function, we first examined the phosphorylation status of p38MAPK upon SnPP-IX treatment. As demonstrated in **Figure 4.6*A***, HO-1 inhibition resulted in a marked increase in p38MAPK phosphorylation. We then assessed the requirement of the p38MAK pathway for the induction of DC maturation elicited by HO-1 inhibition using the pharmacological inhibitor of p38MAPK (SB203580) (English and Cobb, 2002) and examined its effects on SnPP-IX-treated iDC phenotype and function. As shown in **Figure 4.6*B***, inhibition of p38MAPK activity prevented the increase in co-stimulatory molecule expression induced by SnPP-IX treatment of iDCs (MHC class II 43.5 ± 1.0 *versus* 15.9 ± 0.6%, $p < 0.05$, ***panel i***; CD86 40.4 ± 0.9% *versus* 14.8 ± 2.3%, $p < 0.05$, ***panel ii***). Furthermore, p38MAPK inhibition also prevented the enhanced antigen-specific F5 CD8 T cell proliferation mediated by SnPP-IX-treated iDCs (**Figure 4.6*C***, reduction of 2.5-fold at 1 nM NP68, $p < 0.05$; 2-fold at 10 nM NP68, $p < 0.05$; and 1.4-fold at 100 nM, $p < 0.05$, which are comparable to iDCs). To test whether inhibition of p38MAPK activity in SnPP-IX-treated iDCs which resulted in reduced DC maturation phenotype is manifested through altered CREB/ATF1 signalling, DCs were treated with SnPP-IX and SB203580. Western immunoblotting revealed that SnPP-IX treatment resulted in increased CREB/ATF1 phosphorylation (**Figure 4.6*D***). Furthermore, the enhanced CREB/ATF1 phosphorylation was markedly reduced by p38MAPK inhibition. Collectively, these results suggest that HO-1 regulates DC phenotype and function through modulation of the p38MAPK-CREB/ATF1 signalling axis.


A
p-p38
Tubulin
iDC
LPS
SnPP-IX
B (i)
Percentage of cells with high MHC II Expression
60
40
20
0
*
*
iDC
SnPP-IX
SnPP-IX + SB
B (ii)
Percentage of cells with high CD86 Expression
60
40
20
0
*
*
iDC
SnPP-IX
SnPP-IX+ SB
C
150
100
50
0
iDC
SnPP-IX
SnPP-IX + SB
^{3}H-Thy cpm x 10^{-3}
*
*
*
*
*
*
0
1 x 10^{-9}
1 x 10^{-8}
1 x 10^{-7}
NP68 (M)

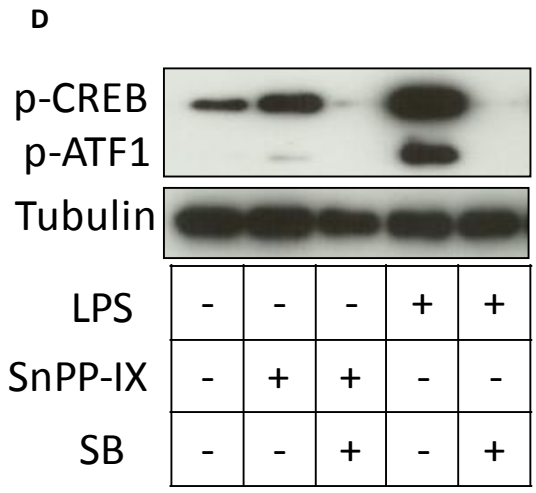
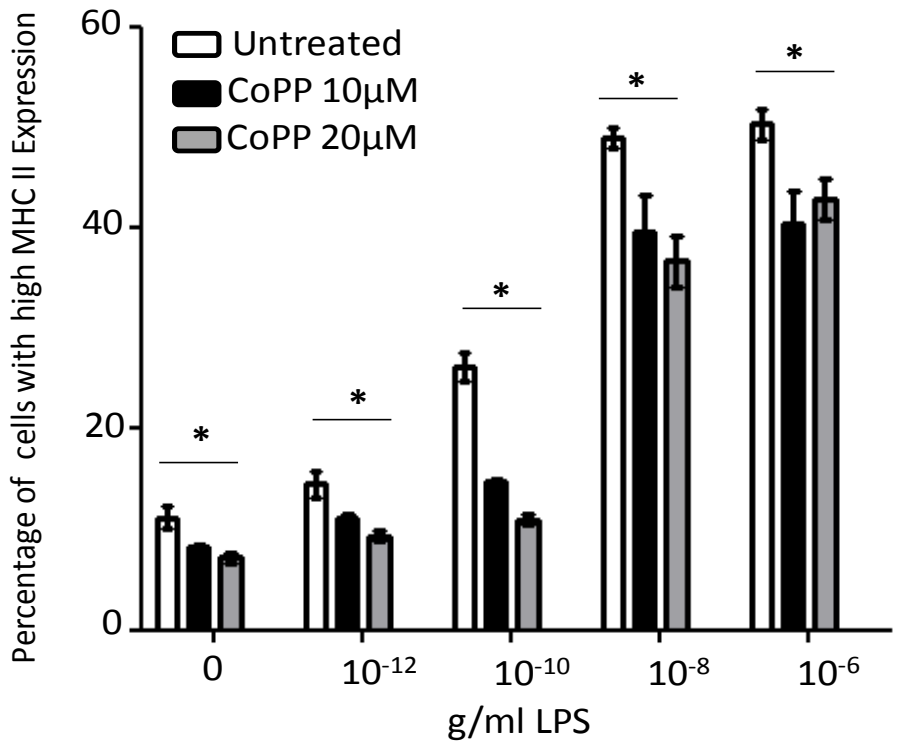


Figure 4.6. Modulation of dendritic cell phenotype and function by HO-1 is mediated through the p38MAPK-CREB/ATF1 pathway. A, Cell lysates generated from iDCs untreated or treated with SnPP-IX (5μM) for 2 h. or with LPS (1 μg/ml) for 30 min, were subjected to SDS-PAGE and levels of phosphorylated p38MAPK (p-p38) and tubulin assessed by Western blotting. **B,** Immature DCs untreated or treated with SnPP-IX (5 μM) for 14 h alone, or treated with p38MAPK activity inhibitor SB203580 (SB, 20 μM) for 48 h along with SnPP-IX (5 μM) for the last 14 h. MHC class II **(i)** and CD86 **(ii)** expression were determined by flow cytometry and presented as percentage of cells expressing high MHC class II or CD86. Data derived from three independent experiments are presented as average percentage ±S.D. (*, $p < 0.05$). **C**, Immature DCs were untreated or treated with SnPP-IX (5 μM) for 14 h, or treated with SB (20 μM) for 48 h along with SnPP-IX (5 μM) for the last 14 h. DCs were then pulsed with increasing concentrations of NP68 antigenic peptide, and co-cultured with F5 CD8 T cells for 72 h. [^{3}H]-Thymidine (^{3}H-Thy) was added for the last 16 h. Proliferation of T cells was determined by scintillation counting of incorporated (^{3}H-Thy). Data are presented as average scintillation counts ± S.D. Statistical significance was assessed using one-way ANOVA. Data are representative of three independent experiments (*, $p < 0.05$). **D**, Cell lysates were generated from iDCs untreated or treated with SnPP-IX (5μM) for 2 h in the presence or absence of SB (20 μM) for 1 h prior to SnPP-IX treatment. These iDCs were subjected to SDS-PAGE and phosphorylation status of CREB and ATF1 (p-CREB and p-ATF1) assessed by Western blotting. Lysates from iDCs treated with SB (20 μM) for 1 h in the presence or absence of LPS (1 μg/ml) for the last 30 min were also included. Tubulin was assessed for equal loading of lanes.

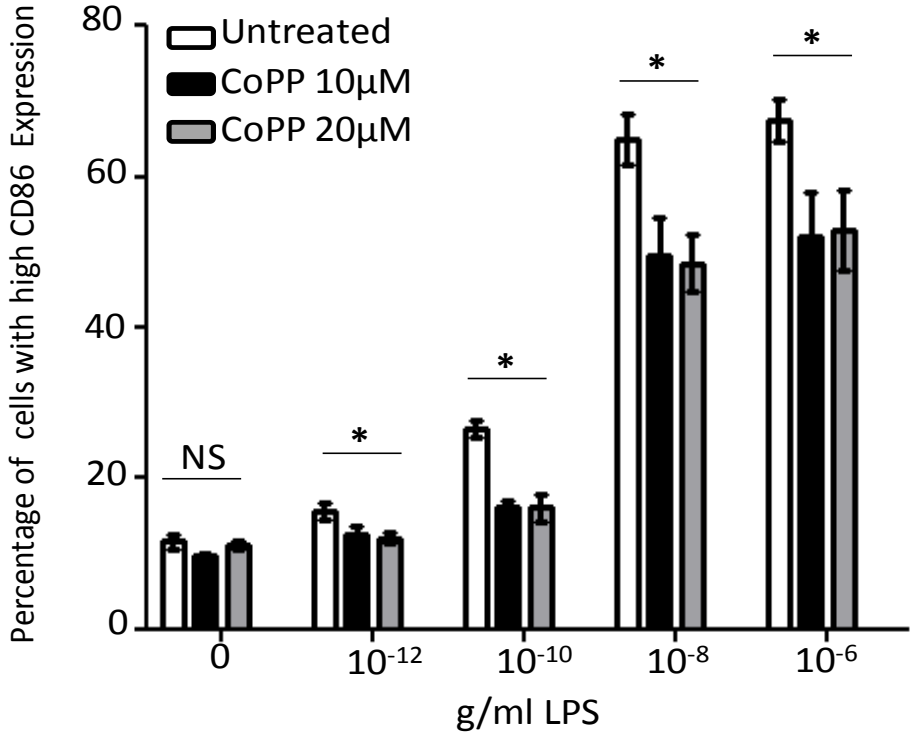
4.2.7 Up-regulation of HO-1 renders DCs refractory to LPS-induced DC signalling and maturation

In order to further test the involvement of HO-1 in the regulation of DC phenotype and function through p38MAPK-CREB/ATF1, we investigated the effect of HO-1 up-regulation on LPS-triggered DC maturation and CREB/ATF1 phosphorylation. Cobalt protoporphyrin (CoPP) can be used to up- regulate the expression of HO-1 (Rosa et al., 2008) and we used it to treat iDCs (at concentrations of 10 µM and 20 µM). We demonstrated that both basal and LPS-induced up-regulation of MHC class II molecules expression was significantly reduced (**Figure 4.7*A panel i***). HO-1 induction also causes significant reduction in LPS-induced up-regulation of CD86 (**Figure 4.7*A panel ii***). Furthermore, CoPP treatment resulted in a significant reduction in LPS-treated DC-mediated antigen-specific F5 CD8 T cell proliferation (**Figure 4.7*B***). In order to evaluate the influence of CoPP treatment on LPS-induced phosphorylation of CREB and ATF1, iDCs were treated with CoPP and stimulated with LPS. Cobalt protoporphyrin treatment markedly reduced CREB/ATF1 phosphorylation in LPS-treated DCs as shown in **Figure 4.7*C***. These results strengthen the evidence for the regulation of DC function by HO-1.

A (i)


Percentage of cells with high MHC II Expression
60
40
20
0
Untreated
CoPP 10µM
CoPP 20µM
*
*
*
*
*
0
10-12
10-10
10-8
10-6
g/ml LPS

A (ii)


Percentage of cells with high CD86 Expression
80
60
40
20
0
Untreated
CoPP 10µM
CoPP 20µM
NS
*
*
*
*
0
10-12
10-10
10-8
10-6
g/ml LPS

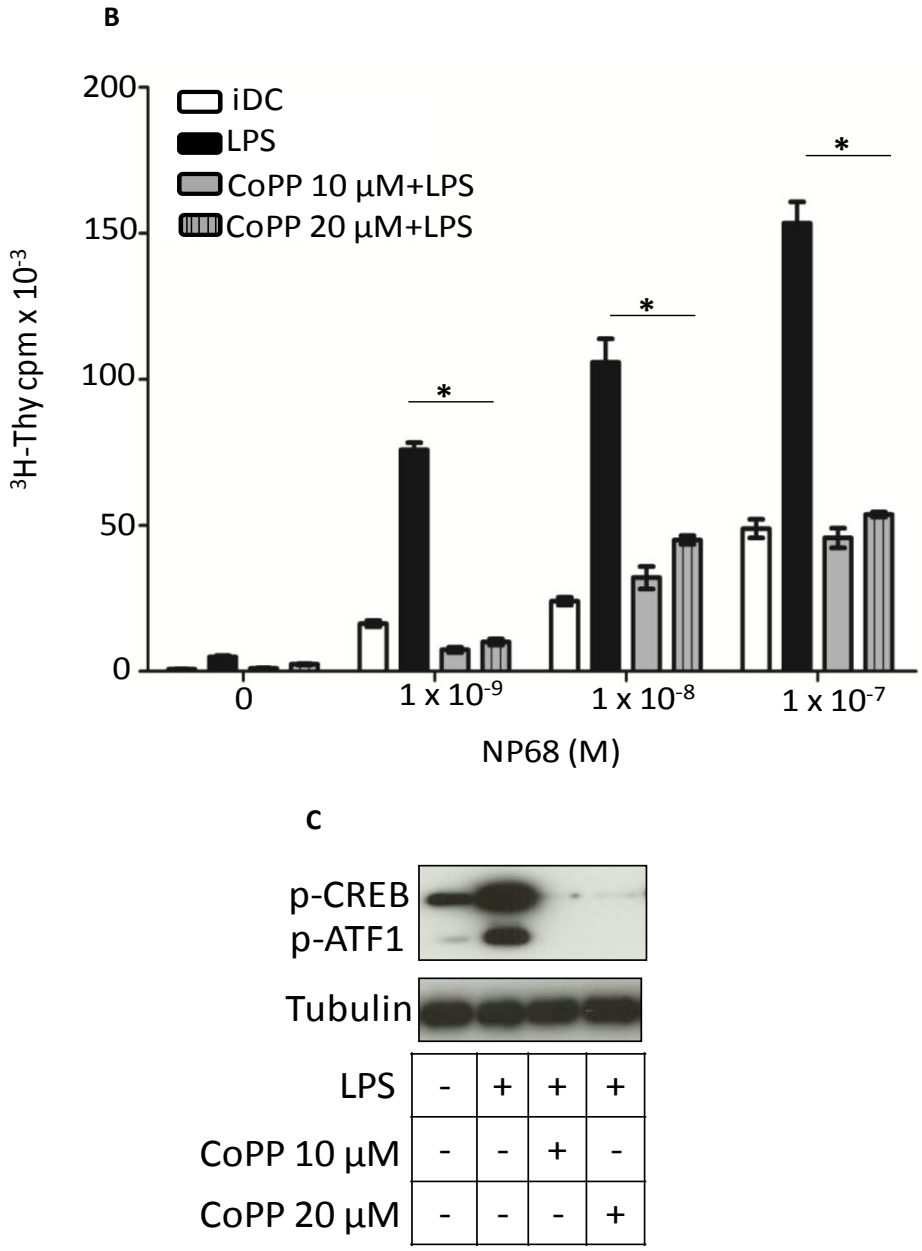


Figure 4.7. Upregulation of HO-1 activity inhibits LPS-induced signalling, phenotypic and functional changes in DCs. A, Immature DCs untreated or treated with HO-1 inducer CoPP (10 µM and 20 µM) for 24 h with or without LPS at indicated concentrations for 18 h. MHC class II **(i)** and CD86 **(ii)** expression were determined by flow cytometry and presented as percentage of cells expressing high MHC class II or CD86. Data derived from three independent experiments are presented as average percentage ±S.D (*, p <0.05; NS, not significant). **B,** Immature DCs were untreated or treated with CoPP (10 µM and 20 µM) for 24 h along with or without LPS (1 µg/ml) for the last 18 h. DCs were then pulsed with increasing

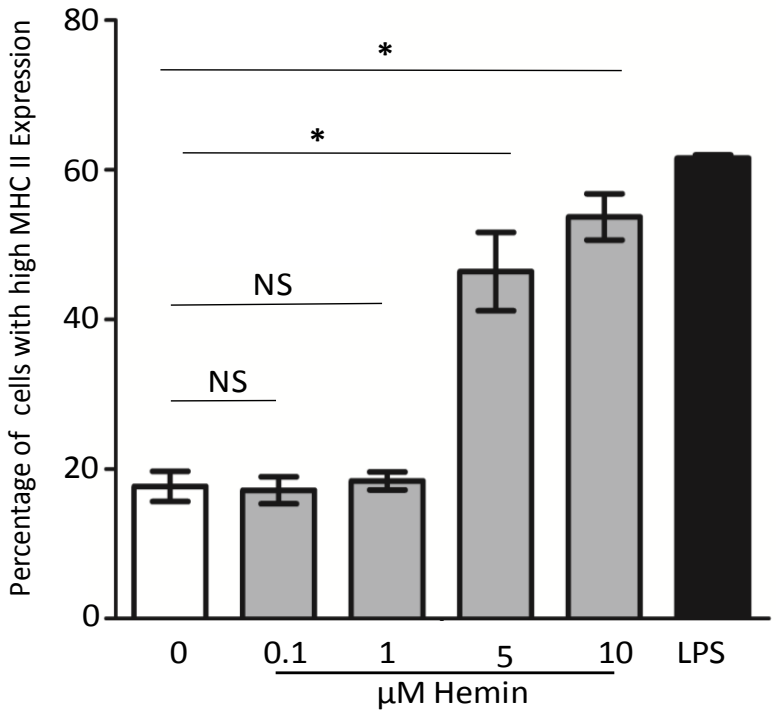
concentrations of NP68 antigenic peptide, and co-cultured with F5 CD8 T cells for 72 h. [^{3}H]-Thymidine (^{3}H-Thy) was added for the last 16 h. Proliferation of T cells was determined by scintillation counting of incorporated (^{3}H-Thy). Data are presented as average scintillation counts ± S.D. Statistical significance was assessed using one-way ANOVA. Data are representative of three independent experiments (*, $p < 0.05$). **C,** Cell lysates generated from iDCs untreated or treated with CoPP (10 μM and 20 μM) for 4 h in combination with LPS (1 μg/ml) for 30 min. Lysates were subjected to SDS-PAGE and phosphorylation status of CREB and ATF1 (p-CREB and p-ATF1) assessed by Western blotting. Lysate from iDCs treated with LPS (1 μg/ml) for 30 min was used as positive control. Tubulin was assessed for equal loading of lanes.

4.2.8 The HO-1 substrate, heme, induces DC functional maturation through p38MAPK-CREB/ATF1 pathway

Inhibition of HO-1 results in intracellular accumulation of the HO-1 substrate, heme (Wagener et al., 2003). When heme (as hemin, the oxidised form of heme), is added to innate immune cells, it can accumulate within the cells (Dang et al., 2011; Hualin et al., 2012). We therefore used hemin to test whether heme can recapitulate the effects of HO-1 inhibition on DC phenotype and function. When iDCs were treated with hemin, the expression of MHC class II and CD86 were up-regulated compared to untreated iDCs (**Figure 4.8*A***, MHC class II 17.7 ± 3.5% (untreated) *versus* 46.4 ± 9.1% (at 5 μM) and 53.7 ± 5.4% (at 10 μM), $p < 0.05$ ***panel i***; CD86, 12.5 ± 0.6% (untreated) *versus* 37.7 ± 9.6% (at 5 μM) and 50.6 ± 4.4% (at 10 μM), $p < 0.05$ ***panel ii***). The mature phenotype observed in hemin-treated DCs was comparable to that of LPS-treated DCs (MHC class II 61.6 ± 0.7%, and CD86 67.2 ± 2.0%). Next we investigated the effect of hemin on DC phago- and endocytic function. Our results revealed that DCs treated with hemin exhibited a reduction in their ability to phagocytose necrotic cells (1.93 ± 0.31 for 5 μM hemin and 1.53 ± 0.41 for 10 μM hemin compared with 3.42 ± 0.21 -fold increase over the baseline for untreated iDCs, $p < 0.05$, **Figure 4.8*B panel i***) and apoptotic cells (2.07 ± 0.61 for 5 μM hemin and 1.55 ± 0.09 for 10 μM hemin compared with 3.54 ± 0.52 -fold increase over the

baseline for untreated iDCs, $p < 0.05$, **Figure 4.8*B panel ii***). A similar reduction was observed in the endocytic capacity of hemin-treated iDCs (**Figure 4.8*C***, 27.5 ± 3.3% for 5 μM hemin and 31.5 ± 7.4% for 10 μM hemin *versus* 62.5 ± 2.7% for iDCs at 15 min, 47.9 ± 3.5% for 5 μM hemin and 49.2 ± 3.2% for 10 μM hemin *versus* 73.1 ± 3.4% for iDCs at 30 min and 60.6 ± 6.1% for 5 μM hemin and 64.7 ± 2.7% for 10 μM hemin *versus* 78.4 ± 1.6% for iDCs at 60 min, $p < 0.05$). Furthermore, hemin-treated iDCs demonstrated an enhanced capacity to stimulate antigen-specific F5 CD8 T cell proliferation compared to untreated iDCs (**Figure 4.8*D***). Finally, we investigated the effects of hemin on the p38MAPK signalling pathway in iDCs. We demonstrated that p38MAPK (**Figure 4.8*E***), CREB, and ATF1 (**Figure 4.8*F***) were hyperphosphorylated in hemin-treated DCs. Taken together, these results suggest that the changes in DC phenotype, function and signalling induced by HO-1 inhibition could be attributable to the effects of the HO-1 substrate, heme.

A (i)


Percentage of cells with high MHC II Expression
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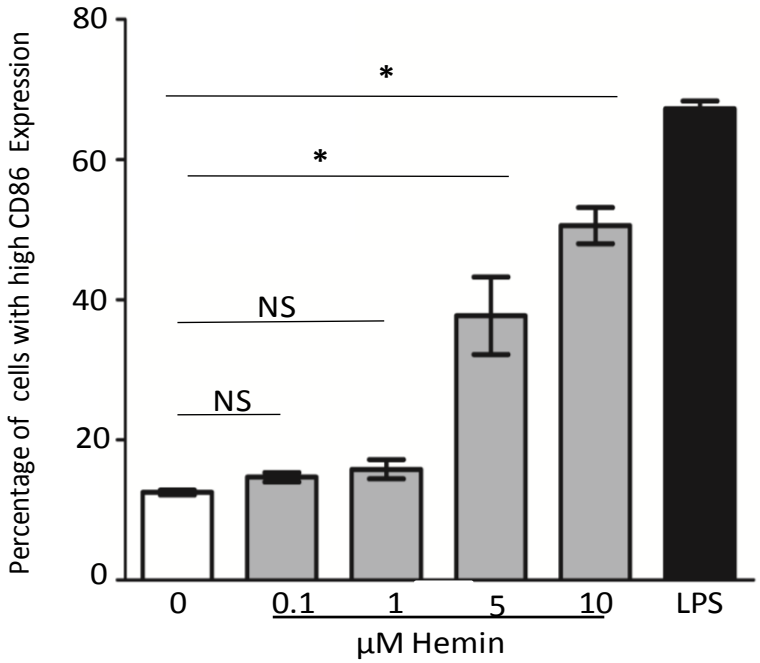
60
40
20
0
*
*
NS
NS
0
0.1
1
5
10
LPS
µM Hemin

A (ii)


Percentage of cells with high CD86 Expression
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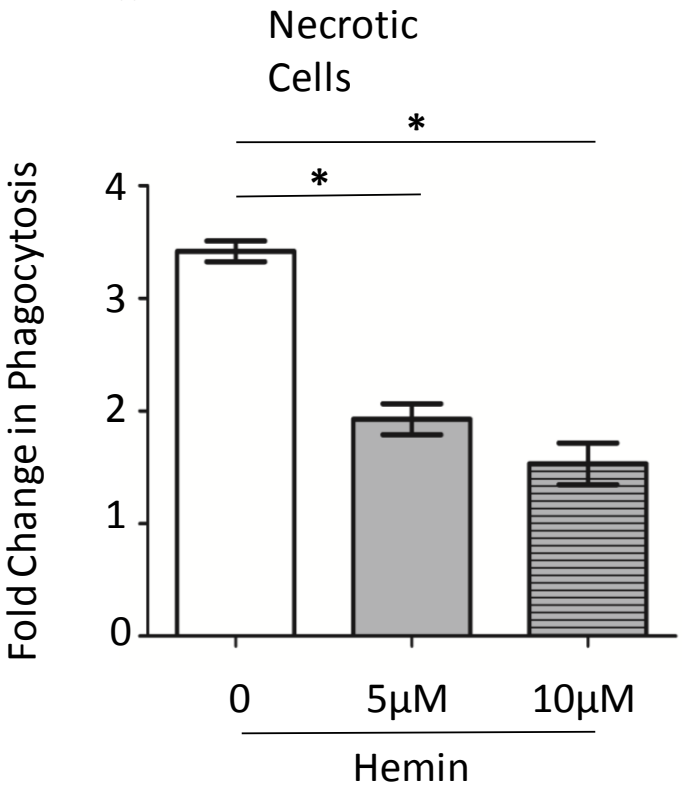
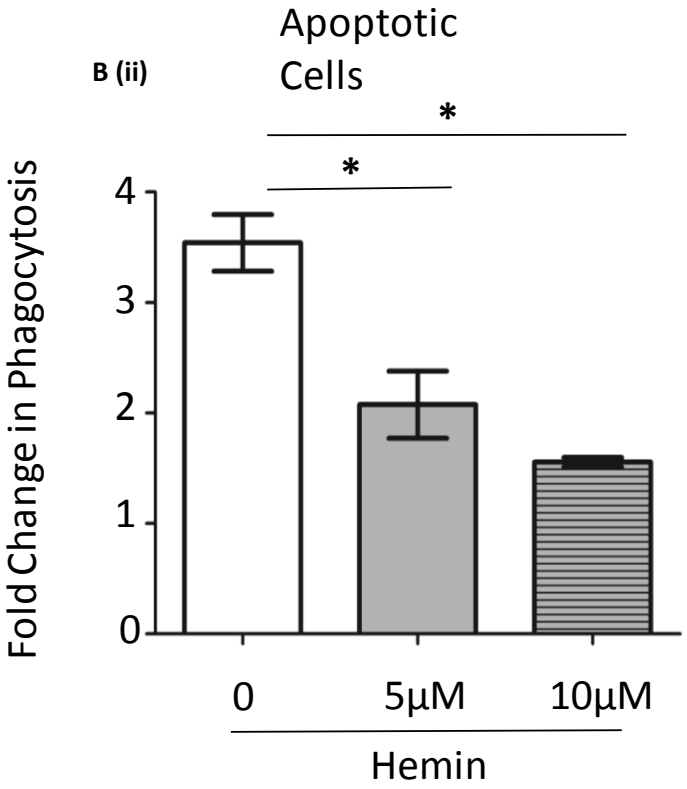
60
40
20
0
*
*
NS
NS
0
0.1
1
5
10
LPS
µM Hemin

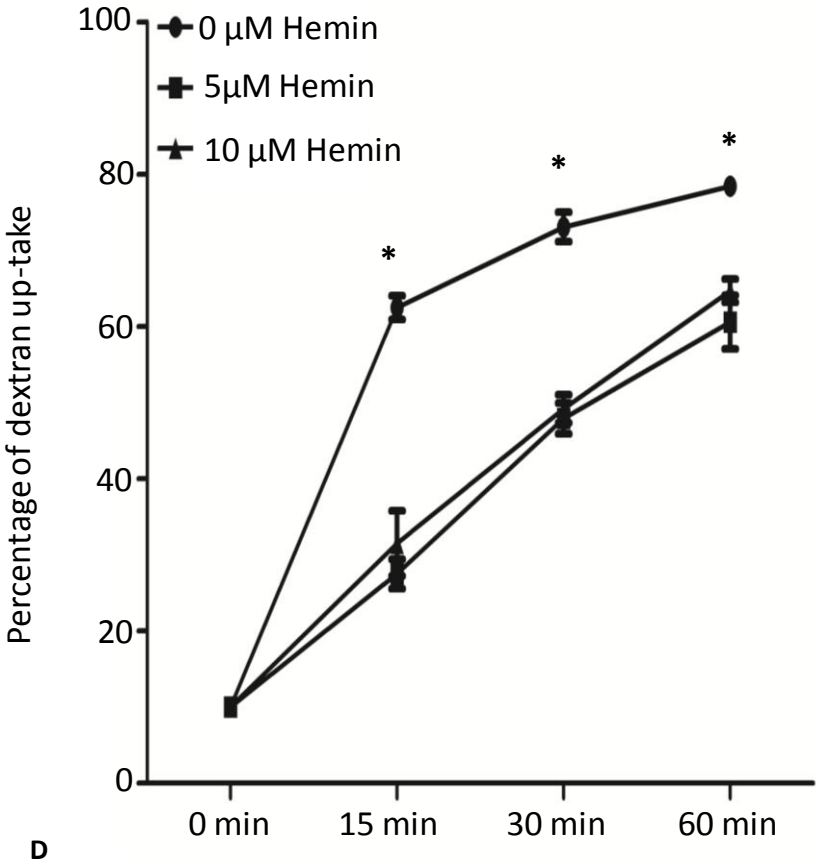
B (i)


Necrotic Cells
*
*
Fold Change in Phagocytosis
4
3
2
1
0
0
5µM
10µM
Hemin

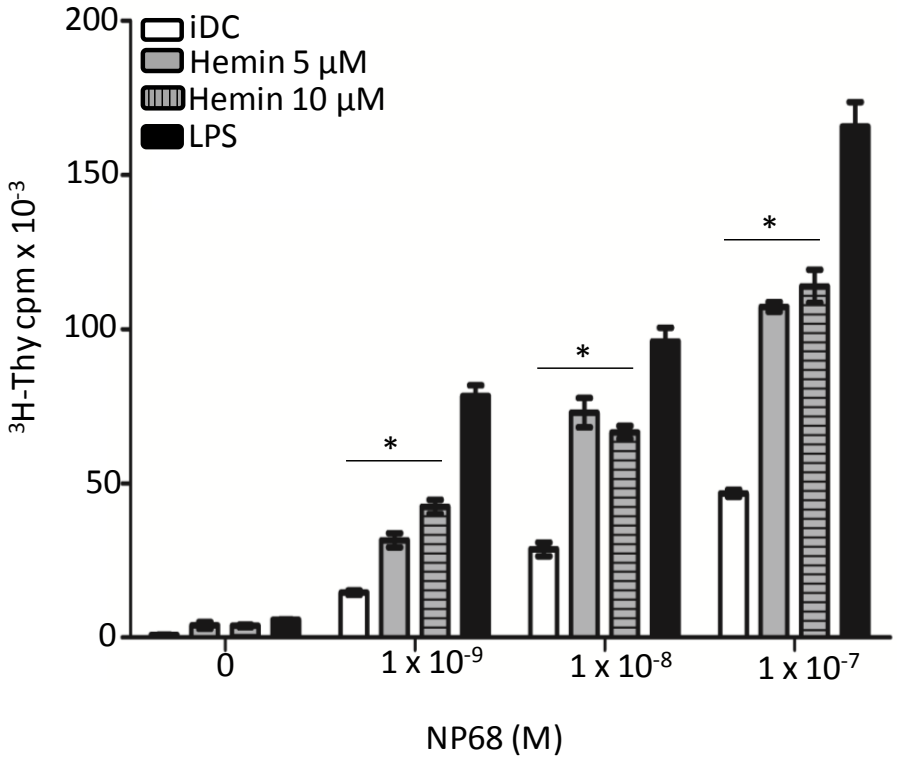
B (ii)


Apoptotic Cells
*
*
Fold Change in Phagocytosis
4
3
2
1
0
0
5µM
10µM
Hemin

C


0 µM Hemin
5µM Hemin
10 µM Hemin
Percentage of dextran up-take
100
80
60
40
20
0
0 min
15 min
30 min
60 min
*
*
*

D


iDC
Hemin 5 µM
Hemin 10 µM
LPS
3H-Thy cpm x 10-3
200
150
100
50
0
0
1 x 10-9
1 x 10-8
1 x 10-7
NP68 (M)
*
*
*

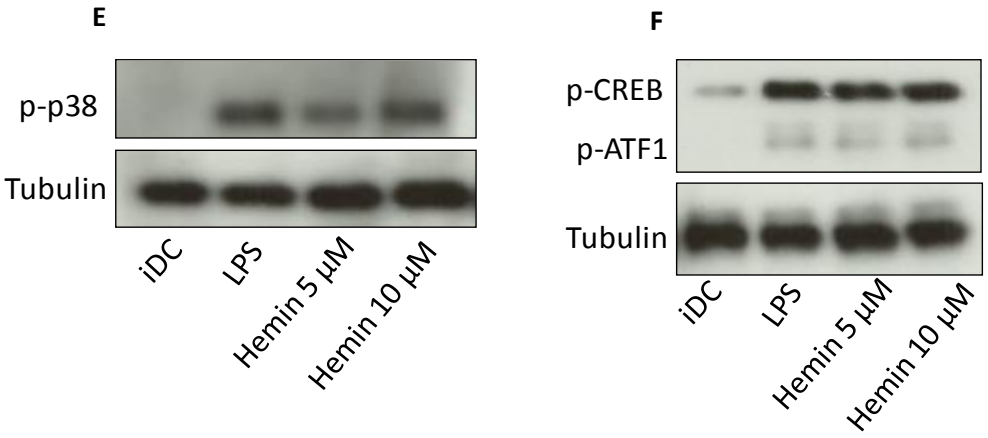


Figure 4.8. HO-1 substrate, heme, induces iDC signalling, phenotypic and functional changes in DCs. A, Immature DCs untreated or treated with hemin (0.1, 1, 5 and 10 µM) or LPS (1 µg/ml) for 14 h. MHC class II (**i**) and CD86 (**ii**) expression were determined by flow cytometry and presented as percentage of cells expressing high MHC class II or CD86. Data derived from three independent experiments are presented as average percentage ±S.D. (*, $p < 0.05$; NS, not significant). **B,** Immature DCs untreated or treated with hemin (5 and 10 µM) for 14 h were co-cultured with CFSE-labelled necrotic Jurkat cells (i) or apoptotic thymocytes (ii) at 37 °C for 2 h. DC phagocytic capacity was measured by flow cytometry as an increase in CFSE levels when compared with corresponding 4°C baseline control samples. Data derived from four independent experiments are presented as average fold changes ± S.E.M. Statistical significance was tested by Mann-Whitney U test (*, $p < 0.05$). **C,** Endocytic capacity was measured by incubating iDCs with $Dextran^{FITC}$ for the indicated time points at 37°C. $Dextran^{FITC}$ uptake by iDCs was assessed by flow cytometry. Data derived from three independent experiments are presented as average percentage of uptake ± S.D. Statistical significance was tested by unpaired Student's t test (*, $p < 0.05$). **D,** Immature DCs were untreated or treated with hemin (5 and 10 µM) or with LPS (1 µg/ml) for 14 h. DCs were then pulsed with increasing concentrations of NP68 antigenic peptide, and co-cultured with F5 CD8 T cells for 72 h. [^{3}H]-Thymidine (^{3}H-Thy) was added for the last 16 h. Proliferation of T cells was determined by scintillation counting of incorporated (^{3}H-Thy). Data are presented as average scintillation counts ± S.D. Statistical significance was assessed using one-way ANOVA. Data are representative of three independent experiments (*, $p < 0.05$). **E,** Cell lysates were generated from iDCs untreated or treated with hemin (5 and 10 µM) or LPS (1 µg/ml) for 30 min. Lysates were subjected to SDS-PAGE and phosphorylation status of p38MAPK (p-p38) assessed by Western blotting. Tubulin was assessed for equal loading of lanes. **F,** Cell lysates were generated from iDCs untreated or treated with (5 and 10 µM) hemin or LPS (1 µg/ml) for 30 min, subjected to SDS-PAGE and phosphorylation status of CREB and ATF1 (p-CREB and

p-ATF1) assessed by Western blotting. Tubulin was assessed for equal loading of lanes.

4.3 DISCUSSION

Heme oxygenase-1 is an Nrf2-inducible gene product that mediates antioxidant and cytoprotective effects resulting in maintenance of cellular redox homeostasis and protects cells from oxidative stress (Abraham et al., 1995; Lee et al., 1996).

In this chapter, we have shown the contribution of HO-1 in the regulation of DC phenotype and immune function. First, we showed that $Nrf2^{-/-}$ iDCs express less HO-1 levels in unstimulated cells. This confirms the requirement for Nrf2 transcriptional activity in for the production of HO-1 protein. Of note, mature DCs express lower levels of HO-1 than immature DCs. Interestingly, $Nrf2^{-/-}$ iDCs which have a reduced expression of HO-1 exhibit a more mature phenotype (as shown in Chapter 3). It is tempting to speculate that reduction in HO-1 is a necessary accompaniment to DC maturation. Furthermore, the presence of significant HO-1 activity may impede the DC maturation signals induced by agents such as LPS. Indeed, inhibition of HO-1 activity in iDCs caused an enhanced maturation phenotype in terms of increased levels of MHC class II and CD86 expression consistent with previous reports (Al-Huseini et al., 2013; Cheng et al., 2010; Figueiredo et al., 2007; Schumacher et al., 2012).

To further delineate the precise role of HO-1 in iDC immune function, we examined the impact of HO-1 inhibition on antigen acquisition. HO-1 inhibition resulted in impaired phagocytic and endocytic function in iDCs. This is most likely to be a consequence of the enhanced maturation status of these DCs rather than direct regulation by HO-1 of these biological processes. A major function of iDCs is to take up antigens via phagocytic and endocytic processes, critical for initiation of T cell immune response and for induction of T cell tolerance (Albert et al., 1998; Steinman, 2003). DCs utilise phagocytic and endocytic mechanisms to acquire

antigens from the extracellular environment and from cells that die (by apoptosis or necrosis) due to infection or normal tissue homeostasis. This is an exceptional function of iDCs and they start to lose such qualities when they mature (Inaba et al., 1993; Sallusto et al., 1995a). As DCs mature, they lose their endocytic and phagocytic capacity, which correlates closely with higher expression of co-stimulatory molecules. Defects in endocytic/ phagocytic function of DCs are associated with systemic lupus erythematosus and Gaucher's disease (Micheva et al., 2006; Monrad et al., 2008).

Both endocytic and phagocytic functions were impaired in SnPP-IX-treated iDCs. It is however unclear whether this effect is due to direct contribution of HO-1 to these biological processes or just due to enhanced maturation status of the cells. It is interesting to note that interference of endocytosis during HO-1 inhibition is much higher in the early phase than the late phase which may suggest a blocking in the initiating step in the dephosphorylation of a group of proteins implicated in endocytosis (Zhao et al., 2010). Examples of these proteins are dephosphins that stimulating the assembly of several large essential endocytic protein complexes (Cousin and Robinson, 2001). Rearrangement of the actin cytoskeleton is involved in the endocytic and phagocytic pathways in DCs (Jutras and Desjardins, 2005; West et al., 2004a) and it is possible that actin polymerisation is impaired in SnPP-IX-treated iDCs. Signalling molecules such as Rho GTPases (Rac, Cdc42 and RhoA) are key players in the regulation of the actin cytoskeleton (Hall, 1998b; Kobayashi et al., 2001). Previous studies have shown that hemin potentiates actin polymerisation and subsequent migration in intestinal epithelial cells and neutrophils (Barcellos-de-Souza et al., 2013; Graca-Souza et al., 2002). Measurement of actin polymerisation (F-actin) and assessing the phosphorylation status of these molecules in bone marrow derived-DCs could shed more light on this issue.

To further explore the role of HO-1 in modulating DC functions, our next approach was to examine iDC-mediated antigen-specific CD8 T cell stimulatory capacity which reflects the DC maturation state. Effector cytotoxic T lymphocyte generation, from naive CD8 T cells, requires strong co-stimulatory signals in addition to TCR signals provided by mature DCs (Chai et al., 1999; Gett et al., 2003; Kemball et al., 2006). Consequently the mature phenotype exhibited by the SnPP-IX-treated iDCs was associated with increased CD8 T cell stimulation capacity. In addition to increased co-stimulation, it is possible that HO-1-inhibited DCs may secrete proinflammatory cytokines that would further influence the quality and magnitude of the CD8 T cell response. Cytokine analysis of the supernatants from HO-1-inhibited iDCs should provide data on this aspect. Immature DCs that have low levels of co-stimulatory molecule expression contribute to immune tolerance by engaging with self-reactive CD8 T cells and causes CD8 T cell anergy or deletion (Kurts et al., 1997a). This implicates HO-1, through its effect on DC co-stimulatory molecule expression, in the maintenance of immune tolerance mediated by iDCs. Interestingly, there is evidence for the requirement of HO-1 function for the expansion of a CD4 subset of immunosuppressive T cells, regulatory T cells (Tregs), the generation of which is dependent on iDCs (Schumacher et al., 2012).

The induction of HO-1 is dependent on the activity of the transcription factor, Nrf2 (Alam et al., 1999). We have previously shown that iDCs which lack Nrf2, display an enhanced maturation phenotype and increased T cell stimulatory capacity that is remarkably similar to DCs treated with SnPP-IX (Aw Yeang et al., 2012) and we showed earlier in this chapter that loss of Nrf2 causes reduction in HO-1 expression. This suggests that lowered HO-1 activity due to loss of Nrf2 underlies the phenotypic and functional changes observed in the Nrf2 deficient iDCs. Our results highlighted the effects of HO-1 induction on counteracting DC phenotypic maturation induced by LPS which is consistent with earlier publications (Listopad et

al., 2007). Up-regulation of HO-1 mRNA and protein by cobalt protoporphyrin was known to be time and dose related (Shan et al., 2006). However, the p38MAPK pathway may mediate this effect as we demonstrated a reduction in CREB and ATF1 phosphorylation upon cobalt protoporphyrin treatment. The mechanism by which HO-1 activity contributes to the maintenance of DCs in an immature state merits further investigation.

One possible mechanism through which HO-1 regulates DC function is through generation of CO and BV by its enzymatic action on its substrate heme (Siow et al., 1999). We hypothesise that upon inhibition of HO-1 activity in iDCs, deprivation of HO-1 products CO and BV in iDCs leads to enhanced maturation and consequently increased T cell stimulatory function. In support of this, previous studies have shown that CO inhibited DC maturation through TLR3 and TLR4 pathways as well as inhibition of secretion of proinflammatory cytokines and induction of alloreactive T cell proliferation (Remy et al., 2009). A recent study on fibroblasts focussing on inducible CO releasing molecules found that CO augments fibroblast survival in hypoxic conditions (Zobi et al., 2013). BV administration reduced neutrophil activity (Freitas et al., 2006) and also protected against LPS-induced shock (Sarady-Andrews et al., 2005), suppressed T cell proliferation, and induced tolerance to cardiac allografts in mice (Yamashita et al., 2004).

In keeping with these findings, we have also demonstrated that induction of HO-1 in DCs using CoPP was associated with lower sensitivity to LPS-induced DC maturation. The potential increase in CO production as a result of higher HO-1 activity in iDCs treated with CoPP may underlie the resistance to TLR4 signalling by LPS. This could be brought about through the interaction of CO with caveolin-1 (cav-1), a structural component of the plasma membrane that when bound to TLR4 in the presence of CO results in inhibition of LPS-induced signalling (Chidlow and Sessa, 2010). Additionally, CO treatment on LPS-treated DCs inhibited CD4 and CD8

T cell activation in the context of soluble antigens (Tardif et al., 2013). Although this effect was not due to altered endocytosis capacity as opposed to our observations, it would be interesting to further dissect these different observations.

Another potential mechanism for HO-1 effects on DC maturation could be through its role in redox homeostasis. Changes in ROS levels have been implicated in many aspects of DC biology including cell maturation and cytokine production (Kantengwa et al., 2003). We demonstrated that HO-1 inhibition increased ROS levels in iDCs possibly due to build up of intracellular heme. Heme can directly induce intracellular ROS generation (Vincent, 1989) and induction of HO-1 was able to reduce high ROS levels in LPS-stimulated DCs (Chauveau et al., 2005). However, lowering ROS levels through vitamin treatment did not alter DC maturation levels induced by HO-1 inhibition, indicating that these changes in DC phenotype and function are not ROS mediated. Consistent with the lack of ROS involvement in mediating these changes in iDCs, similar findings were observed in the previous chapter which demonstrated that resetting elevated ROS levels in Nrf2-deficient DCs did not reverse the dysregulated DC phenotype and function (Al-Huseini et al., 2013).

The molecular pathways that are utilised by HO-1 to modulate DC function, was a focus of our investigations. We provide evidence for the involvement of the p38MAPK-CREB/ATF1 signalling axis in mediating the effects of HO-1 on DC function. Studies that used pharmacological inhibition of p38MAPK activity, have demonstrated the importance of this pathway in DC co-stimulatory molecules up-regulation induced by LPS (Nakahara et al., 2006).

This suggests that in iDCs, HO-1 may exert anti-inflammatory and tolerogenic effects. An inflammatory stimulus like LPS should switch off HO-1 expression to fully initiate effective immune responses. However, this effect might be limited to DCs

due to their particular role in responding to pathogens while other parenchymal cells require an increased expression of HO-1 in order to protect against inflammation (Wiesel et al., 2000). As LPS signals through the TLR4 receptor and subsequently activates the p38MAPK signalling pathway (An et al., 2002), elucidating the effects HO-1 has on this pathway may highlight functional changes when HO-1 levels or its metabolites are altered.

In the current study, p38MAPK activity inhibition prevented the mature DC phenotype in SnPP-IX-treated iDCs. Furthermore, p38MAPK activity inhibition also produced a marked reversal in the enhanced DC-mediated antigen-specific CD8 T cell proliferation observed in SnPP-IX-treated iDCs. In addition, the downstream effectors of p38MAPK, CREB and ATF1 were also activated in SnPP-IX-treated iDCs as revealed by their hyperphosphorylated state, which could be reduced by the p38MAPK activity inhibitor. These results highlight the role for p38MAPK-CREB/ATF1 activity in mediating HO-1 effects on DC function. The exact molecular basis for p38MAPK-CREB/ATF1 activation in HO-1 inhibited DCs is not known but one possibility could be due to interaction of intracellular heme with receptors that signal through this pathway. We tested this possibility by treating DCs with heme and demonstrated that not only did heme activate the p38MAPK-CREB/ATF1 pathway but also recapitulated the other phenotypic and functional effects of HO-1 inhibition in DCs (enhanced co-stimulatory receptor expression, enhanced CD8 T cell stimulatory capacity and reduced endo- and phagocytic capability).

Heme is synthesised in all human nucleated cells and its levels are regulated through synthesis of heme which is counterbalanced via degradation of heme by HO-1. Synthesis and degradation of heme are thus closely linked (Abraham and Kappas, 2008). Heme synthesis involves a series of enzymatic reactions localised in the mitochondrion and cytoplasm of cells. Depending on cell and tissue types,

heme requirements can vary significantly. The most rapid rates of heme synthesis occur in bone marrow erythroid cells and in liver hepatocytes. Due to the incorporation of heme into the heme proteins hemoglobin and cytochrome P450, heme is synthesized in very high quantities in these respective organs. It has been postulated that most mammalian cells contain a “free” or “uncommitted” heme pool, serving both precursor and regulatory functions in the synthesis and regulation of heme levels.

Heme derived from denatured heme proteins other than hemoglobin is probably degraded locally by HO while heme derived from hemoglobin degradation is reused for re-synthesis of haemoglobin (Abraham et al., 1988; Ponka, 1999; Wagener et al., 2003). Increased heme levels leads to a potential amplifier of the inflammatory response and is a characteristic feature of diseases with increased hemolysis or widespread cell damage (Jeney et al., 2002). Heme is quite hydrophobic and readily enters cell membranes. Brief incubation of immune cells with micromolar concentrations of hemin (5-10 μM) triggered the oxidative burst and the production of reactive oxygen species was directly proportional to the concentration of hemin added to the cells (Graca-Souza et al., 2002). However, prolonged incubation with higher concentration of heme will induce stimulation of HO-1 causing increases in its cytoprotective activity and subsequent reduction of heme levels (Soares et al., 2009).

While Nrf2 is a potent positive regulator of the HO-1 gene (Alam et al., 1999), bach1 is a transcriptional repressor of HO-1 (Kitamuro et al., 2003). Bach1 binds in conjunction with a small Maf protein to tandem repeats of the antioxidant response element (ARE) and quenches the target gene expression (Ishikawa et al., 2005). When HO-1 is inhibited, heme will build up leading to increase ROS levels which causes redox conformational modification in Bach1 rendering it unable to

bind ARE. This will enhance the binding of Nrf2 to the ARE and promote transcription of HO-1 (Ishikawa et al., 2005).

In our system we timed the experiment to inhibit HO-1 activity by SnPP-IX but at the same time not allowing heme builds up to induce HO-1 expression which could subsequently mask the end results.

Heme has been shown to engage and activate TLR4 thereby potentially activating signalling pathways including the p38MAPK-CREB/ATF1 axis (Figueiredo et al., 2007). We therefore speculate that elevated level of heme in HO-1-inhibited DCs activates TLR4. However, there is a kinetic difference in the induction of p38MAPK-CREB/ATF1 pathway between heme build up due to HO-1 inhibition and hemin-treated DCs. The former condition showed increase phosphorylation of p38MAPK, CREB and ATF1 after 2 h while the latter showed that in a much shorter time (30 min) with decreasing phosphorylation at 2 h.

It has been shown that macrophages which are pre-exposed to free heme lose their ability to respond, and become desensitized to a subsequent challenge by other TLR4 agonists like LPS (Otterbein et al., 2003). This immunoregulatory effect could be attributed to the induction of HO-1 and in particular to the production of CO. Additionally, there is a different activation profile for heme and LPS which might in part be related to the association of these molecules at different binding sites of the TLR4 receptor complex. Furthermore, heme polymers like hemozoin and β-hematin have been shown to activate the innate immune system through TLR9 while heme itself wasn't able to activate TLR9 (Figueiredo et al., 2007).

This could be tested by examining whether the altered phenotype, function and signalling seen in HO-1-inhibited DCs is abolished when HO-1 is inhibited in TLR4 deficient DCs.

Given that multiple DC functions are modulated by HO-1, it would be interesting to determine the cytokine expression profile of DCs treated with hemin may shed further light in order to examine its role in heme-induced dysregulation in DC immune function. Indeed, previous studies in DCs and other cell types have implicated the role of HO-1 in modulating cytokine production (Barcellos-de-Souza et al., 2013; Graca-Souza et al., 2002; Tardif et al., 2013).

HO-1 is being considered as potential future clinical approach following successful therapeutic targeting of a variety of animal models of inflammatory conditions or disorders involving the immune system, including organ transplantation, autoimmunity and allergy (Ryter et al., 2006) as it improves graft survival through local anti-inflammatory and modifying T cell functions as well as inducing immunosuppressive molecules in transplantation (Blancou et al., 2011; Soares et al., 1998). Therefore it is critical to understand the details of HO-1-mediated regulation of DC function.

Taken together these findings along with other publications, HO-1 can be considered as a promising immunosuppressive molecule in organ transplantation In atherosclerotic diseases, modulation of HO-1 induction has been anticipated to be a potential therapeutic target and *in vitro* screening assay for anti-atherosclerotic agents (Wu et al., 2006).

Our observations provide further details and insights into the role of HO-1 in DC biology and could therefore inform the design of future pharmacological strategies that aim to modulate DC function in the therapy of immune diseases.

CHAPTER FIVE

FINAL DISCUSSION

5.1 Introduction

Dendritic cells have a central role in the immune system and have been recognised as key communicating sentinels at the interface between the innate and adaptive immune responses. Maturation of DCs is a process that has a significant impact on both innate and acquired immune responses, whereby antigen-capturing, poorly immunogenic iDCs are transformed into highly immunogenic antigen-presenting mDCs which migrate from peripheral tissues to lymphoid organs subsequently activating naive T cells.

Precise regulation of intracellular signalling pathways is vital for normal DC maturation and function. Studies have shown that modulation of these pathways can modify the immune response elicited by DCs. Stimulation of PRRs on the DC surface following pathogen infection or tissue damage results in the activation of members of each of the major MAPK subfamilies — p38MAPK, ERK and JNK subfamilies. MAPKs activation, in conjunction with the activation of NF-κB, induces the expression of multiple genes that collectively regulate the inflammatory response.

DC function is subject to the influence of its intracellular redox potential as oxidative stress has been shown to be detrimental to normal physiological functioning. Generally, mammalian cells have evolved a highly regulated cell defence system that affords protection against the harmful effects of exogenous and endogenous chemical and oxidant insults. Natural or pharmacological manipulation of the activity and levels of cellular antioxidants may have vital effects on their functions including regulation of genes that are implicated in the chronic inflammatory disease process (Chen and Kunsch, 2004). Redox-sensitive transcription factor Nrf2 activity is a principal cellular defence system against oxidative stress.

It is clear that understanding how the transcription factor Nrf2, and its transcribed genes, influence DC function through their effects on redox homeostasis and signalling pathways in DCs is required before targeting of this system for therapeutic purposes becomes a viable strategy.

This thesis dissects the role of two major redox regulators; the transcription factor Nrf2 and its transcribed protein HO-1 in controlling DC intracellular signalling and function.

5.2 Nrf2 modulates DC immune function through regulation of p38MAPK-CREB/ATF1 signalling

The first part of this study demonstrated that Nrf2 deficiency in mouse bone marrow derived-iDCs resulted in increased DC proliferation, ROS production, enhanced iDC co-stimulatory receptor expression, and increased iDC-mediated antigen-specific CD8 T cell stimulatory capacity.

Nrf2 controls the expression of a large number of genes that enable a coordinated protective response to oxidative stress. These Nrf2-responsive genes regulate the transcriptional induction of a battery of drug-detoxifying enzymes and proteins include glutathione, heme oxygenases, γ-Glutamylcysteine synthetase, thioredoxin, Glutamate—cysteine ligase catalytic subunit, glutathione reductase, superoxide dismutase, catalase, UDP-glucuronyl transferases, and quinone reductases. The proteins encoded by Nrf2-responsive genes function as direct antioxidants, conjugate to and detoxify natural and xenobiotic electrophiles, metabolise free radicals, recognize DNA damage, drive GSH synthesis, regulate the proteasome and molecular chaperones, and inhibit inflammation (Itoh et al., 2010; Slocum and Kensler, 2011). The critical role of Nrf2-regulated enzymes in the detoxification of a wide range of chemicals, drugs, and toxins has been well-established (Cederbaum, 2009; Chan et al., 2001; Osburn and Kensler, 2008).

Nrf2 is correlated to differentiation, growth, proliferation, apoptosis and it is thought that Nrf2 has evolved from an original role in haematopoiesis with the regulation of cell differentiation from early lineages (Li et al., 2012).

Loss of Nrf2 was found to enhance lymphocyte proliferation as well as renal mesangial cell proliferation which showing acute and chronic glomerulonephritis is consistent with multiple inflammatory episodes similar to that seen in systemic lupus erythematosus (SLE) (Li et al., 2004; Ma et al., 2006). In the current study, the findings of increase DC proliferation and enhanced expression of anti-apoptotic protein Bcl-2 might also contribute to this pathology. Previous publications have shown that accelerated proliferation together with poor detoxification exacerbated tumor transformation susceptibility in Nrf2 deficient mice (Ma and He, 2012).

Enhanced co-stimulatory receptor expression in iDCs has been shown to cause a break in peripheral T cell tolerance, through the prolongation of T cell activation and enhancement of adaptive immune responses (Hawiger et al., 2001). Therefore, in the absence of infection, it would be reasonable to propose that Nrf2 deficient iDCs (instead of instructing autoreactive T cell deletion or anergy) could present self-antigens to CD8 T cells in such a context that may result in the induction of inappropriate CD8 T cells activation, adaptive immune responses, and autoimmunity. Indeed results from our lab presented evidences that the CD8 T cells activated by the $Nrf2^{-/-}$ iDCs develop effector functions (Aw Yeang et al., 2012). The role of Nrf2 in the regulation of T cell tolerance and autoimmunity is further emphasised by the fact that aged Nrf2 deficient mice manifest enhanced lymphoproliferation and autoimmune-like symptoms (Ma et al., 2006).

Results from our lab demonstrated that T cells from $Nrf2^{-/-}$ mice manifest signs of low level T cell activation with a small but consistent reduction in CD62L expression when compared with their $Nrf2^{+/+}$ counterpart presumably through interaction with DCs in the lymph nodes (Aw Yeang et al., 2012). These findings raise the possibility

that Nrf2 could play an important role in the maintenance of peripheral T cell tolerance by iDCs.

The rationale for targeting the Nrf2 system is therefore a promising strategy for the therapy of autoimmune diseases.

Evidence has shown that the intracellular redox status of the DC has a profound impact on its functions such as DC activation, maturation, cytokine production, and immunosenescence (Kim et al., 2008; Verhasselt et al., 1999b; Williams et al., 2008). Therefore, strategies based on therapeutically manipulation of these redox balance regulators are achieving enormous consideration.

Oxidative stress occurs when the production of ROS exceeds their catabolism. However, the term stress is an imprecise reference to a restricted range of ROS signalling that runs from adaptive to maladaptive cellular changes (Nathan and Cunningham-Bussel, 2013). When ROS generation overwhelms the cellular buffering capacity as shown in **Figure 5.1**, this may lead to modulating or utilizing of intracellular signalling pathways from cell surface receptors like MAPKs and NF-κB, in association with direct damage, resulting in cell death or pathological processes (Nakamura et al., 1997a). Oxidative damage, caused by defective defences and increased generation of ROS, has been implicated in the pathogenesis of both aging and of autoimmune disease (Droge, 2002). In the context of DCs, ROS may act as a "danger" signal and could induce phenotypic and functional maturation of DCs (Kantengwa et al., 2003; Rutault et al., 1999). Consistent with this premise, treatment with both vitamins C and E antioxidants results in DC resistance to phenotypic and functional changes induced by high ROS levels (Tan et al., 2005).

Global deletion of Nrf2 in mice lowers the redox potential, impairs induction of cellular antioxidant defences, and enhances vulnerability and severity to several inflammatory disorders including asthma, emphysema, fibrosis and colitis (Kensler et al., 2007; Rangasamy et al., 2004; Rangasamy et al., 2005).

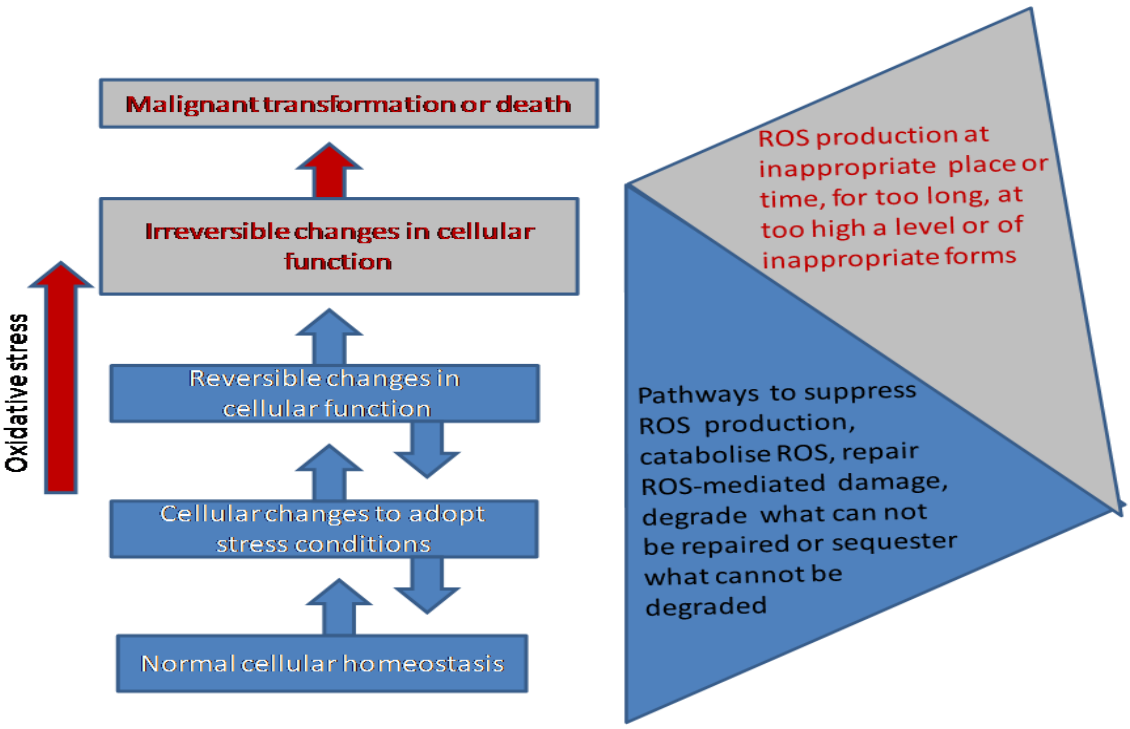


Figure 5.1. ROS effect on homeostasis adopted from Nathan & Cunningham, 2013

Glutathione is a cellular buffer for ROS (Kim et al., 2007). Work done by our group has shown that GSH was not responsible for altered phenotype and function observed in Nrf2$^{-/-}$ iDCs (Aw Yeang et al., 2012). In this study, we demonstrate that elevated ROS does not contribute towards the development of altered phenotype and function observed in Nrf2$^{-/-}$ DCs (Al-Huseini et al., 2013). This suggests that the effects of long term high ROS levels on cellular function may be through conformation changes in cell kinases and transcription factors thus potentially influencing their association with other cellular proteins. However, our approach to reduce ROS levels using vitamins C and E reduced elevated ROS levels lower than Nrf2 sufficient DCs but this was ineffective in reversing the altered DC phenotype and function. Other approaches for reducing ROS levels by ROS scavengers other than antioxidant vitamins, like edaravone (Mikawa et al., 2005), may be worth investigating in future studies for further confirmation of the above results.

ROS have various inhibitory or stimulatory roles in NF-κB signalling (Morgan and Liu, 2011). NF-κB proteins are sensitive to oxidation leading to a modification that decreases DNA binding; it is therefore possible that modulation of the redox state of NF-κB could represent a post-translational control mechanism for this factor (Toledano and Leonard, 1991).

Our results showed that loss of Nrf2 did not affect basal NF-κB activity but it impedes activity of upstream elements of the NF-κB pathway (phosphorylation of p65 and IκBα degradation). These results suggest redox modification of upstream IκB kinases due to prolonged high ROS levels exposure could lead to aberrant NF-κB signalling. This needs to be tested by normalising ROS levels in $Nrf2^{-/-}$ DCs and examining NF-κB activation. However, inhibiting NF-κB pathway did not reverse the altered DC phenotype and function in the absence of Nrf2 (Aw Yeang et al., 2012). This suggests that any alterations in NF-κB signalling in these DCs is not part of the mechanism that leads to altered DC phenotype and function in of $Nrf2^{-/-}$ DCs.

Although the three MAPKs (ERK1/2, JNKs, p38MAPK) are found to be activated i.e. hyperphosphorylated in $Nrf2^{-/-}$ iDCs, only inhibition of p38MAPK pathway was able to reverse the altered DC phenotype and function. This pathway also contributed to HO-1 mediated alteration in DC biology. These findings highlight a pivotal role for p38MAPK in the regulation of DC maturation and antigen-specific CD8 T cell stimulation. This is consistent with findings in other cells such as endothelial cells wherein pharmacological activation of Nrf2 was able to reduce endothelial cell activation through inactivation of p38MAPK by suppressing an upstream activator MKK3/6 and by enhancing the activity of the negative regulator MKP-1 (Zakkar et al., 2009). This may suggest a reciprocal relation between Nrf2 and p38MAPK. However, we cannot say for certain that the phenotype exhibited by the Nrf2 deficient DCs is only mediated through p38MAPK as HDACs has also been shown to contribute to DC function dysregulation.

5.3 Heme oxygenase-1 regulates dendritic cell function through modulation of p38MAPK-CREB/ATF1 signalling

Nrf2 binds to antioxidant-responsive elements (AREs) and this induces the expression of stress-responsive genes that governs the production of a wide range of cytoprotective enzymes and proteins, including HO-1 (Alam and Cook, 2003). HO-1 contributes to defence mechanisms against oxidative stress inducing agents such as heme/haemoglobin, metals, various cytokines, and endotoxin. Overexpression of HO-1 in cells might, therefore, protect against oxidative stress produced by certain of these agents particularly heme and haemoglobin by catalysing their degradation to biliverdin/bilirubin and CO which both have antioxidant properties (Abraham, 2003; Baranano et al., 2002; Otterbein Le Fau - Bach et al.; Sammut et al., 1998).

In chapter 4, we have shown the contribution of HO-1 to DC phenotype and function. We found that interfering with HO-1 activity resulted in increased ROS levels and DC phenotypic and functional changes similar to that induced by inflammatory stimuli e.g. LPS. It was concluded that this effect was also mediated through p38MAPK-CREB/ATF1. The ability of HO-1 to inhibit the maturation state of DCs has been linked to the protective effect of Treg cells on pregnancy outcome (Schumacher et al., 2012).

Studies of HO-1 deficiency in humans and knockout mice have revealed a protective role for HO-1 in inflammation (Rushworth and O'Connell, 2004). Human HO-1 deficiency leads to severe growth retardation with spontaneous development of a chronic inflammatory pathology characterized by increased serum IgM and blood leukocyte count, accumulation of polymorphonuclear (PMN) cells, monocytes and macrophages in the spleen as well as in non-lymphoid tissues and wide-spread oxidative tissue injury (Soares et al., 2009; Yachie et al., 1999). Additionally, HO-1

knockout mice showed reduced stress defence capacity and were hypersensitive to cytotoxicity caused by hemin and hydrogen peroxide (Poss and Tonegawa, 1997).

Extensive randomized clinical trials have been done using HO-1 inhibitors to control serum bilirubin levels in patients with hereditary porphyria (Dover et al., 1991), liver disease (Berglund et al., 1990), Crigler-Najjar type I syndrome (Galbraith et al., 1992), and in newborns with ABO incompatibility where SnPP-IX can, in appropriate doses, moderate the postnatal rate of increase of plasma bilirubin levels and diminish the intensity of hyperbilirubinemia in treated babies (Kappas et al., 1988) or in glucose 6-dehydrogenase deficiency where HO-1 inhibitor administration preventively or therapeutically entirely supplanted the need for phototherapy to control hyperbilirubinemia (Valaes et al., 1998).

Cytoprotective effect of HO-1 can be attributed to the end product of its activity like CO and BV. It will be interesting to test the effect of CO and BV on $Nrf2^{-/-}$ iDC phenotype and function. These DCs may show low levels of CO and BV and their supplementation with these molecules might result in restore altered DC phenotype and function observed in $Nrf2^{-/-}$ iDCs.

The release of heme from damaged cells and tissues is believed to be higher in trauma and haemorrhage or in diseases such as malaria and autoimmune haemolytic anaemia. Hence, heme can be involved in the Danger model concept which proposes that endogenous molecules from damaged tissues activate the immune system and cause inflammation (Mortensen et al., 2008). These danger signals would be able to initiate effective immune responses through DC maturation and enabling these DCs to activate naive T cells. Furthermore, heme catalyzes the formation of ROS, resulting in oxidative stress (Jeney et al., 2002; Vercellotti et al., 1994).

We demonstrated, upon hemin treatment, the DCs exhibited increased co-stimulatory molecule expression with enhanced DC-mediated T cell stimulation. However, this effect induced by exogenouse hemin administration is potentially time-limited as prolonged exposure to heme will ultimately induce HO-1 activity and this will have anti-inflammatory effect by subsequently reducing co-stimulatory expression as shown in **Figure 5.2.**

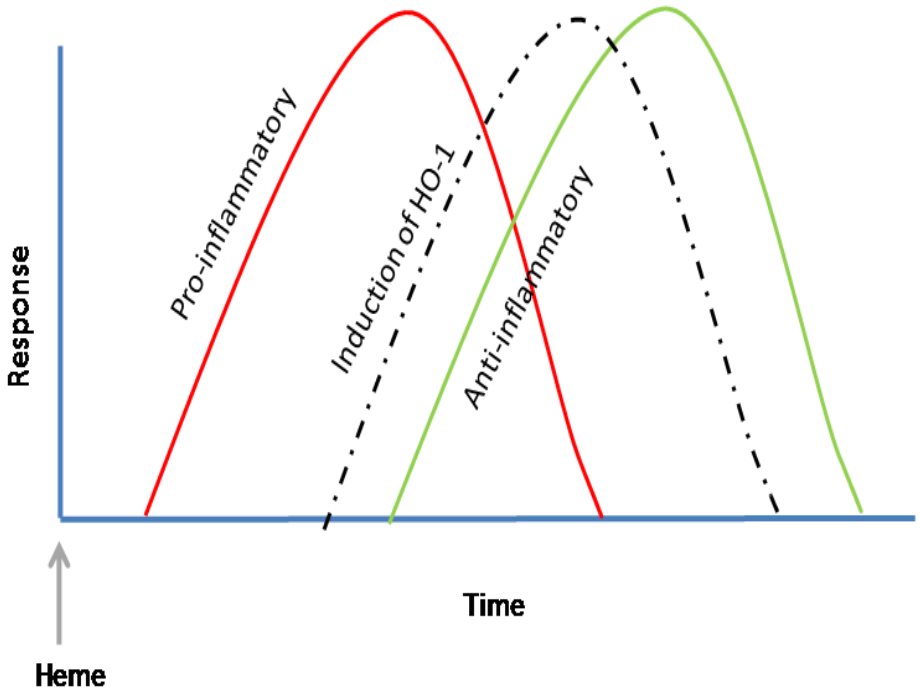


Figure 5.2. Regulation of immune response by the heme/HO-1 system. Initially free heme induces a pro-inflammatory response followed by induction of HO-1 leading to anti-inflammatory response attributable to HO-1. (adopted from Soares et al 2009) .

Observations from this study are based on *in vitro* approach as DCs are generated from mice bone-marrow progenitors. Therefore DCs from an *in vivo* model may be required to exclude the contribution of the *ex vivo* culturing environment on DC biology. However, this approach is not without limitations, with the most important one being the difficulty in getting enough DC numbers (both from peripheral blood and spleens) for functional studies.

Additionally, it would be best to have a DC specific Nrf2 knockout alone without the involvement of other cells especially T cells. Pharmacological and gene silencing of Nrf2 or HO-1 through siRNA knockdown in DCs may be another approach that would reaffirm our findings.

In summary, we have studied modulation of multiple aspects of DC phenotype and function by two important redox regulators, Nrf2 and HO-1. **Figure 5.3** summarises the key results from our study and includes key findings from other studies. It highlights the contribution of intracellular signalling pathways and molecular disruption to altered DC phenotype and function in the absence of these redox regulators. From these studies we have identified several pathways that could be targeted in order to modulate DC function. However further studies are required to convert these discoveries into a viable therapeutic reality.

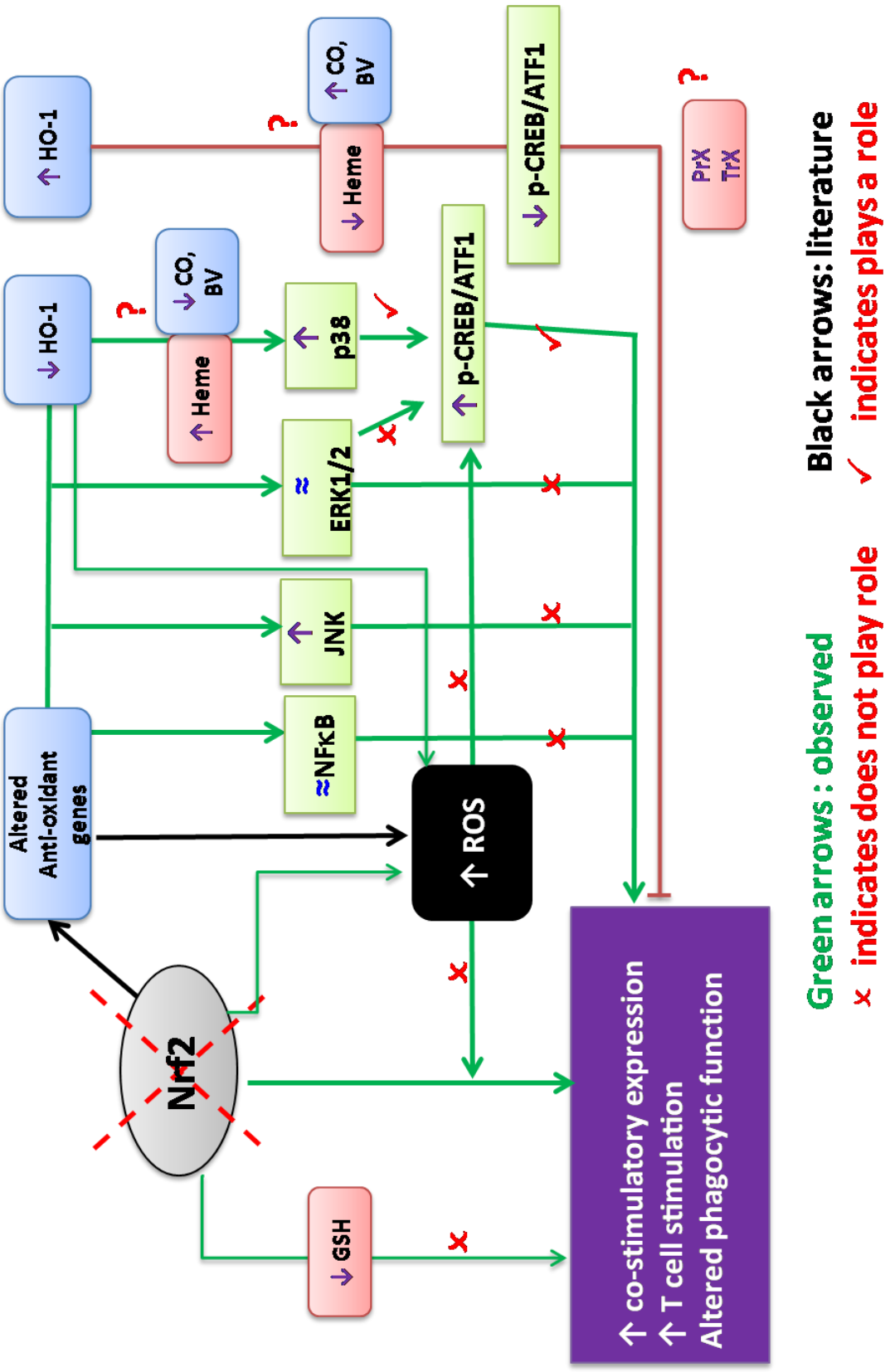


Figure 5.3. Schematic summary of thesis results

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